DISTRIBUTED-IN-SPACE GLUTATHIONE CONJUGATION KINETICS OF ETHACRYNIC ACID IN LIVER

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmaceutical Sciences
University of Toronto

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ABSTRACT

The factors affecting the hepatocellular glutathione (GSH) conjugation of ethacrynic acid (EA), a model substrate, in the formation of the glutathione adduct EA-SG were assessed for their influence on local GSH conjugation rates from the inlet to the outlet of the liver. These included plasma protein binding, sinusoidal uptake, spontaneous and enzyme-catalyzed conjugation, co-substrate (GSH) availability and EA-SG efflux at canalicular and sinusoidal membranes and their zonation within the acinus. The spontaneous coupling of EA with GSH was rapid, whereas the in vitro conjugation catalyzed by the glutathione S-transferases (GSTs) in rat liver was even faster. The conjugation rates greatly exceeded the EA uptake rate by saturable and non-saturable processes in isolated rat hepatocytes. When these in vitro data were viewed with respect to events in whole liver (single-pass perfused rat liver), transport and not GSH conjugation was found to be rate-limiting in the rapid conversion of EA to EA-SG. In studies with isolated, enriched periportal and perivenous hepatocytes, EA uptake and EA-SG efflux were functionally similar among hepatocytes throughout the acinus. However, a shallow and increasing (portal to venous) GST activity gradient was observed in rat liver. When these zonal, uptake and metabolic in vitro data were utilized to predict EA disposition in liver perfusions in the presence and absence of bovine serum albumin (BSA), a series compartment model was found to be most consistent with data obtained in the absence of albumin. The model predicted both a temporal and zonal depletion of GSH among hepatocytes and a change in the rate-limiting step with increasing perfusion time from uptake to co-substrate availability. The model was less capable of predicting the influence of high albumin protein binding on
uptake and metabolism, unless a role of BSA in facilitating drug uptake was assumed. This investigation showed for the first time that, in addition to metabolic enzymes, flow and substrate binding, other important factors such as zonal transport, co-substrate, and GSH conjugate efflux further influence the distributed-in-space processing of acceptor substrates in GSH conjugation.
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I would like to express my sincere gratitude to my supervisor, Dr. K. Sandy Pang, for giving me the inspiration, motivation and opportunity to experience research. I am deeply thankful for her guidance throughout the years.

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TABLE OF CONTENTS

Title................................................................. i

Abstract............................................................ ii

Acknowledgments................................................... iv

Table of Contents................................................. v

Abbreviations and Terms........................................... x

List of Tables...................................................... xiv

List of Figures.................................................... xv

Chapters

1. General Introduction........................................... 1

1.1 Biological Aspects of Hepatic Glutathione Conjugation........ 2
   1.1.1 Hepatic glutathione homeostasis............................. 2
   1.1.2 Glutathione conjugation reactions........................... 4
   1.1.3 Glutathione S-transferases.................................... 7
   1.1.4 Membrane associated proteins in eicosanoid and
        glutathione metabolism.......................................... 8
   1.1.5 Mechanism of Glutathione S-transferases...................... 9
   1.1.6 Role of glutathione conjugation in detoxication,
        toxicity and carcinogenesis.................................... 10
   1.1.7 Role of glutathione conjugation in drug resistance........ 11
   1.1.8 Role of glutathione conjugation in formation of
        endogenous substances.......................................... 12
   1.1.9 Hepatic glutathione conjugate export......................... 12
   1.1.10 Hepatic glutathione conjugate catabolism.................... 15
1.1.1 Zonation of hepatic glutathione conjugation ............................... 16
  1.1.1.1 Zonation of substrate uptake or in situ formation rates ......................... 17
  1.1.1.2 Zonation of glutathione S-transferases .......................................... 19
  1.1.1.3 Zonation of glutathione ................................................................. 20
  1.1.1.4 Zonation of glutathione conjugate efflux transporters .......................... 21

1.2 Integrative View of GSH Conjugation in Whole Liver ......................... 21
  1.2.1 Traditional models ................................................................. 22
  1.2.2 Models that incorporate co-substrate ............................................. 25
  1.2.3 Models that incorporate zonation ................................................... 25
  1.2.4 Models that incorporate co-substrate and zonation ............................. 26

1.3 Ethacrynic Acid (EA) as a Model Compound to Study the Kinetics of Hepatic Glutathione Conjugation .................................................. 26
  1.3.1 Pharmacology ................................................................. 26
  1.3.2 Disposition in vivo ............................................................... 27
  1.3.3 Plasma protein binding ......................................................... 28
  1.3.4 Metabolism in vitro ........................................................... 28
  1.3.5 Transport of ethacrynic acid and metabolites .................................... 31
  1.3.6 Toxicology ................................................................. 31
  1.3.7 EA as a model drug to study glutathione conjugation ............................ 33

2. Statement of Purpose of Investigation .............................................. 34

  2.1 Hypotheses ................................................................. 36
  2.2 Objectives ................................................................. 36

3. Bimolecular Glutathione Conjugation Kinetics of Ethacrynic Acid in Rat Liver: *In Vitro* and Perfusion Studies ........................................... 37

  3.1 Abstract ................................................................. 38
  3.2 Introduction ............................................................... 38
  3.3 Methods ................................................................. 40
  3.3.1 Chemicals ............................................................... 40
4.3.4 Metabolism of EA by PP and PV rat hepatocytes ........................................ 79
4.3.5 Analysis ........................................................................................................... 80
4.3.6 GST activity of PP and PV hepatocyte cytosols .............................................. 80
4.3.7 PP and PV cell lysate ...................................................................................... 81
4.3.8 Kinetic modeling of EA disposition by PP and PV hepatocytes ....................... 81
4.3.9 Immunoblot Analysis ...................................................................................... 83
4.3.10 Statistical analysis ......................................................................................... 85
4.4 Results ............................................................................................................... 85
  4.4.1 Biochemical characterization of PP and PV hepatocytes and lysates ............... 85
  4.4.2 Concentration-dependent uptake of EA by zonal hepatocytes ....................... 86
  4.4.3 Cellular GSH concentration and GSH conjugation rates of EA by PP and PV hepatocytes ................................................................. 86
  4.4.4 Fitting of data to kinetic model ..................................................................... 91
  4.4.5 GST activities in cytosolic fractions of PP and PV hepatocytes and lysates .... 93
  4.4.6 GSTs in PP and PV hepatocytes and lysates ................................................. 93
  4.4.7 Mrp2 in PP and PV hepatocytes ................................................................... 93

4.5 Discussion .......................................................................................................... 99
4.6 Statement of Significance of Chapter 4 ............................................................... 103
4.7 Acknowledgments ............................................................................................... 103

5. Distributed-in-Space Modeling of Glutathione Conjugation of Ethacrynic Acid in the Perfused Rat Liver ................................................................. 104
  5.1 Abstract ............................................................................................................ 105
  5.2 Introduction ....................................................................................................... 106
  5.3 Methods ........................................................................................................... 108
    5.3.1 Chemicals .................................................................................................... 108
    5.3.2 Rat liver perfusion ..................................................................................... 108
    5.3.3 Analyses ..................................................................................................... 108
    5.3.4 GSH histochemistry .................................................................................. 109
    5.3.5 Correlation of data to hepatic clearance models ....................................... 109
    5.3.6 Physiological modeling ............................................................................ 111
      5.3.6.1 BSA-free perfusion ............................................................................ 111
# ABBREVIATIONS AND TERMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>APM</td>
<td>Aminopeptidase M</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>$B^A_{\text{max}}$</td>
<td>Maximum concentration of high affinity EA binding site on BSA</td>
</tr>
<tr>
<td>$B^B_{\text{max}}$</td>
<td>Maximum concentration of low affinity EA binding site on BSA</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSP</td>
<td>Bromosulfophthalein</td>
</tr>
<tr>
<td>BSP-SG</td>
<td>Bromosulfophthalein-glutathione conjugate</td>
</tr>
<tr>
<td>BUM</td>
<td>Bumetanide</td>
</tr>
<tr>
<td>cMOAT</td>
<td>Canalicular multispecific organic anion transporter</td>
</tr>
<tr>
<td>$\hat{C}$</td>
<td>Unbound logarithmic average concentration</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CG</td>
<td>Cysteinylglycine</td>
</tr>
<tr>
<td>$CL_b{\text{EA-SG}}$</td>
<td>Biliary intrinsic clearance for EA-SG</td>
</tr>
<tr>
<td>$CL_{\text{deg}}{\text{GSH}}$</td>
<td>GSH degradation clearance</td>
</tr>
<tr>
<td>$CL_{\text{efflux}}^{\text{EA-SG}}$</td>
<td>Net EA-SG efflux clearance into the extracellular compartment</td>
</tr>
<tr>
<td>$CL_h$</td>
<td>Hepatic clearance</td>
</tr>
<tr>
<td>$CL_{\text{int.all}}$</td>
<td>Overall intrinsic clearance</td>
</tr>
<tr>
<td>$CL_s{\text{EA-SG}}$</td>
<td>Sinusoidal transmembrane clearance for EA-SG</td>
</tr>
<tr>
<td>$CL_{\text{ves}}^{\text{EA-SG}}$</td>
<td>Net EA-SG efflux clearance into the vesicular sequestration compartment</td>
</tr>
<tr>
<td>CuOOH</td>
<td>Cumene hydroperoxide</td>
</tr>
<tr>
<td>CYS</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCE</td>
<td>Dichloroethylene</td>
</tr>
<tr>
<td>DCPBA</td>
<td>4-(2,4-dichlorophenoxy)-butyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DDP</td>
<td>Dipeptidyl dipeptidase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNP-SG</td>
<td>Dinitrophenyl-glutathione conjugate</td>
</tr>
<tr>
<td>EA</td>
<td>Ethacrynic acid</td>
</tr>
<tr>
<td>EA-CG</td>
<td>Ethacrynic acid cysteinylglycine conjugate</td>
</tr>
<tr>
<td>EA-CYS</td>
<td>Ethacrynic acid cysteine conjugate</td>
</tr>
<tr>
<td>EA-NAC</td>
<td>Ethacrynic acid N-acetylcysteine conjugate</td>
</tr>
<tr>
<td>EA-SG</td>
<td>Ethacrynic acid glutathione conjugate</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol -bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>EHBR</td>
<td>Eisai hyperbilirubinemic rats</td>
</tr>
<tr>
<td>E</td>
<td>Extraction ratio</td>
</tr>
<tr>
<td>$E_{ss}$</td>
<td>Steady-state extraction ratio</td>
</tr>
<tr>
<td>$E_2$-17β-G</td>
<td>Estradiol-17β-glucuronide</td>
</tr>
<tr>
<td>$f_u$</td>
<td>Fraction unbound in plasma</td>
</tr>
<tr>
<td>GCS</td>
<td>γ-glutamylcysteine synthetase</td>
</tr>
<tr>
<td>GGT</td>
<td>γ-glutamyltranspeptidase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine synthetase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>4HNE</td>
<td>4-hydroxynonenal</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Spontaneous GSH conjugation rate constant</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>$K_{m}^{EA}$</td>
<td>Michaelis-Menten constant for EA in the enzymatic glutathione conjugation</td>
</tr>
<tr>
<td>$K_m^{GSH}$</td>
<td>Michaelis-Menten constant for reduced glutathione in the enzymatic GSH conjugation of EA</td>
</tr>
<tr>
<td>$K_{m}^{uptake}$</td>
<td>Michaelis-Menten constant for facilitated uptake of EA</td>
</tr>
<tr>
<td>$k_{offA}$</td>
<td>Dissociation rate constant for high affinity EA binding site on BSA</td>
</tr>
<tr>
<td>$k_{offB}$</td>
<td>Dissociation rate constant for low affinity EA binding site on BSA</td>
</tr>
<tr>
<td>$k_{onA}$</td>
<td>Association rate constant for high affinity EA binding site on BSA</td>
</tr>
<tr>
<td>$k_{onB}$</td>
<td>Association rate constant for low affinity EA binding site on BSA</td>
</tr>
<tr>
<td>$K_{syn}$</td>
<td>Zero-order glutathione synthesis rate</td>
</tr>
<tr>
<td>$LTC_4$</td>
<td>Leukotriene C$_4$</td>
</tr>
<tr>
<td>MAPEG</td>
<td>Membrane associated proteins in eicosanoid and glutathione metabolism</td>
</tr>
<tr>
<td>MCT</td>
<td>Monocarboxylate transporter</td>
</tr>
<tr>
<td>MGST</td>
<td>Microsomal glutathione S-transferase</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug resistance-associate protein</td>
</tr>
<tr>
<td>NAC</td>
<td>$N$-acetylcysteine</td>
</tr>
<tr>
<td>NAPQI</td>
<td>$N$-acetyl-$p$-quinone-imine</td>
</tr>
<tr>
<td>NAT</td>
<td>$N$-acetyltransferase</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na-K-2CL co-transporter</td>
</tr>
<tr>
<td>Oatp</td>
<td>Organic anion transporting polypeptide (rat)</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic cation transporter</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>$P_{aff}$</td>
<td>Linear transmembrane clearance for EA</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PP</td>
<td>Periportal</td>
</tr>
<tr>
<td>PS$_{in}$</td>
<td>Influx permeability surface area product</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------</td>
</tr>
<tr>
<td>PS_{out}</td>
<td>Efflux permeability surface area product</td>
</tr>
<tr>
<td>PV</td>
<td>Perivenous</td>
</tr>
<tr>
<td>Q</td>
<td>Perfusate flow rate</td>
</tr>
<tr>
<td>Q_{bile}</td>
<td>Bile flow rate</td>
</tr>
<tr>
<td>rcGSHT</td>
<td>Rat canalicular reduced glutathione transporter</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPT</td>
<td>Renal proximal tubule</td>
</tr>
<tr>
<td>rsGSHT</td>
<td>Rat sinusoidal reduced glutathione transporter</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylsulfate</td>
</tr>
<tr>
<td>TALH</td>
<td>Thick ascending loop of Henle</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered-saline</td>
</tr>
<tr>
<td>TCA</td>
<td>Taurocholate</td>
</tr>
<tr>
<td>TR:</td>
<td>Transport deficient Wistar rats</td>
</tr>
<tr>
<td>V_{max}</td>
<td>Maximum velocity</td>
</tr>
<tr>
<td>V_{metab,max}</td>
<td>Maximum enzymatic glutathione conjugation rate for EA</td>
</tr>
<tr>
<td>V_{uptake,max}</td>
<td>Maximum facilitated uptake of EA</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1-1</td>
<td>Summary of rat glutathione S-transferases (GSTs)</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>Summary of multidrug resistance-associated proteins (MRPs)</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>1-3</td>
<td>Rat GST activities towards ethacrynic acid (EA)</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>3-1</td>
<td>Inhibition of $[^{14}C]$EA uptake by organic anions</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>3-2</td>
<td>Parameters for the physiologic kinetic model of EA disposition in perfused rat liver</td>
<td>66</td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td>Summary of dose recovery during rat liver perfusions</td>
<td>67</td>
</tr>
<tr>
<td>4</td>
<td>4-1</td>
<td>Marker enzyme activities of PP and PV hepatocytes and lysates</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>4-2</td>
<td>Summary of EA (200 μM) disposition by PP and PV rat hepatocytes</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>4-3</td>
<td>Parameters for the kinetic model of EA disposition in isolated PP and PV rat hepatocytes</td>
<td>94</td>
</tr>
<tr>
<td>5</td>
<td>5-1</td>
<td>Parameters for the series-compartment model of EA disposition in perfused liver</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>5-2</td>
<td>Summary of predicted steady-state extraction ratios of EA in the presence and absence of BSA</td>
<td>121</td>
</tr>
<tr>
<td>6</td>
<td>6-1</td>
<td>Basolateral uptake systems for organic anions in rat hepatocytes</td>
<td>140</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 1</td>
<td>1-1</td>
<td>Structure of glutathione (GSH) and conjugation with an electrophile</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>1-2</td>
<td>Reactions catalyzed by glutathione S-transferases (GSTs)</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>1-3</td>
<td>Intrahepatic mercapturate formation</td>
</tr>
<tr>
<td>Chapter 1</td>
<td>1-4</td>
<td>Sequential metabolism of ethacrynic acid (EA)</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-1</td>
<td>Mass spectrometric analysis of synthesized EA-SG and EA-CG</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-2</td>
<td>Mass spectrometric analysis of synthesized EA-CYS and EA-NAC</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-3</td>
<td>Stability of EA and its thiol metabolites at pH 7.4 and 3.2</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-4</td>
<td>Repetitive UV spectral analysis of the spontaneous conjugation of EA with GSH</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-5</td>
<td>GSH conjugation of EA by erythrocyte containing perfusate</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-6</td>
<td>Scheme for the hepatic disposition of EA and EA-SG in the physiological kinetic model</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-7</td>
<td>HPLC analysis of EA and its thiol metabolites</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-8</td>
<td>Spontaneous and rat liver cytosol-enhanced GSH conjugation of EA</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-9</td>
<td>Time-dependent uptake of [14C]EA by isolated rat hepatocytes</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-10</td>
<td>Concentration-dependent uptake of [14C]EA by isolated rat hepatocytes</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-11</td>
<td>Protein binding of EA to BSA at equilibrium</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-12</td>
<td>Viability of the erythrocyte-free perfused rat liver and relationship between biliary EA-SG excretion and bile flow</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-13</td>
<td>Metabolism of various concentrations of EA in the single-pass perfused rat liver. Formation of EA-SG and depletion of hepatic GSH</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-14</td>
<td>Hepatic GSH/GSSG content following 90 min single-pass perfusion with various concentrations of EA</td>
</tr>
<tr>
<td>Chapter 3</td>
<td>3-15</td>
<td>Steady-state extraction ratio of EA and total EA-SG formation rate as a function of logarithmic average EA concentration</td>
</tr>
</tbody>
</table>
3-16 Control analysis of the effect of $V_{max}$ for metabolism and $V_{max}$ for uptake on the steady-state extraction ratio of EA .......................... 68

Chapter 4

4-1 Model of GSH conjugation of EA in isolated rat hepatocytes ............... 84
4-2 Concentration-dependent uptake of [¹⁴C]EA by isolated PP and PV rat hepatocytes ........................................ 87
4-3 Metabolism of EA by isolated PP rat hepatocytes ................................. 88
4-4 Metabolism of EA by isolated PV rat hepatocytes ................................. 89
4-5 GST activities towards EA and CDNB by PP and PV hepatocyte cytosols and dual-digitonin-pulse perfusion lysates ......................... 95
4-6 Immunoblot analysis of GST Ya and Yb2 in hepatocyte cytosols .......... 96
4-7 Immunoblot analysis of GST Ya and Yb2 in dual-digitonin-pulse perfusion lysates ................................................. 97
4-8 Immunoblot analysis of Mrp2/cMoat and CYP1A2 in crude membrane fractions derived from PP and PV rat hepatocytes .......... 98

Chapter 5

5-1 “Series compartment” model for hepatic EA disposition during protein-free perfusion ......................................................... 112
5-2 Scheme for protein binding disequilibrium of EA in the sinusoids during BSA perfusions ....................................................... 116
5-3 Bile flow rate during BSA and protein-free rat liver perfusions ......... 117
5-4 Effect of BSA on the hepatic extraction of EA, formation of EA-SG and hepatic glutathione ......................................................... 120
5-5 Fits of the series compartment model to data in absence of BSA ....... 123
5-6 Fits of the series compartment model with sinusoidal binding disequilibrium to data in the presence of BSA .......................... 124
5-7 Predicted temporal gradients in sinusoidal [EA] and [GSH] among zonal compartments with EA perfusions in the absence of BSA ........ 125
5-8 Predicted temporal gradients in sinusoidal [EA] and [GSH] among zonal compartments with EA perfusions in the presence of BSA .... 126
5-9 Stability of GSH in 4% paraformaldehyde at 4 °C ............................... 127
CHAPTER 1

GENERAL INTRODUCTION
1.1 BIOLOGICAL ASPECTS OF HEPATIC GLUTATHIONE CONJUGATION

Glutathione (γ-L-glutamyl-L-cysteinylglycine, GSH) is the most abundant, low molecular weight, thiol present in mammalian cells (0.2 -10 mM) (Anderson, 1998) that serves in a variety of well recognized, physiological and biochemical functions. First, GSH and its oxidized form, GSSG, participate in the maintenance of protein-thiol redox status, thus regulating protein structure and function (Cotgreave, 1998). Second, GSH acts as an important cellular antioxidant that protects the cell membrane, cytoskeleton, DNA and other cellular structures from the deleterious effects of reactive oxygen species (ROS) (Ketterer, 1998). Third, GSH is involved in reduction of ribonucleotides to deoxyribonucleotides, illustrating another of many important cellular functions of this tripeptide. In a physiological sense, GSH serves as a storage and transport form for cysteine within the body (Ookhtens and Kaplowitz, 1998). Because cysteine rapidly autooxidizes to cystine, a reaction which is accompanied by the formation of oxygen radicals, intercellular cysteine exchange occurs through transport within the bloodstream in the form of GSH.

1.1.1 Hepatic GSH Homeostasis

The liver contains a high level of GSH (~6 μmol/g or 8-10 mM) (Fernandez-Checa et al., 1993). Within the hepatocyte, 90% of the GSH can be found in the cytosol while 10% is compartmentalized in the mitochondria (Meredith and Reed, 1982). A small fraction of cellular GSH is localized in the endoplasmic reticulum where it functions in protein folding (Ferrari and Söling, 1999). Hepatocytes do not take up intact GSH from the blood stream (where concentrations of ~15 μM are observed). Rather, the tri-peptide is synthesized in the cytosol from precursor amino acids (see review by Lu, 1999). For the first of two ATP consuming steps, γ-glutamyl-L-cysteine is produced from glutamate and cysteine by the enzyme, γ-glutamylcysteine synthetase (GCS), whereas for the second step, further addition of glycine is catalyzed by the enzyme, GSH synthetase. GSH synthesis is rate-limited by GCS activity and
the availability of cysteine. Dietary cysteine and its oxidized form, cystine, are taken up by hepatocytes by the amino acid transport systems, ASC and Xc, respectively. Alternatively, hepatocytes can produce cysteine from methionine via the transsulfuration pathway. This pathway is the source of 30-50% of cysteine within hepatocytes (Penttilä, 1990). Mitochondrial GSH, originating from the cytosol (Martensson et al., 1990), is transported into the organelle by dicarboxylate and 2-oxoglutarate carriers (Chen and Lash, 1998) in a process which sustains mitochondrial GSH at slightly higher concentrations (~11 mM) than the cytosol (Soboll et al., 1995). The concentration of GSH in the nucleus has been much debated (Bellomo et al., 1992; Briviba et al., 1993) but it appears to be similar to the cytosolic concentration (Soboll et al., 1995). Although the mechanism of GSH transport into the nucleus remains uncertain, there is evidence that Bcl-2 may play a role (Voehringer et al., 1998).

The turnover of hepatic GSH is approximately 2-3 hours (Lu, 1999), and this is mainly due to GSH efflux into plasma and bile. As a consequence, the liver is the principle source of GSH in the blood (Ookhtens and Kaplowitz, 1998) while excretion of GSH into bile is responsible for the non-bile salt dependent bile flow (Ballatori and Truong, 1989). Sinusoidal efflux of GSH has been well characterized at the functional level (Garci-Ruiz et al., 1992; Lu et al., 1994), but there remains controversy over the putative protein(s) involved. A sinusoidal GSH transporter (rsGSHt) that was putatively cloned (Yi et al., 1995) was later determined to be an E. coli gene artifact since full function of the protein could not be reproduced (Li et al., 1998b). The organic anion transporter 1 (oatp1) cloned from rat liver (Jacquemin et al., 1994) has been shown to take up solutes from blood into hepatocytes with GSH as the counter ion (Li et al., 1998a). Despite the demonstrable efflux of GSH by oatp1, this protein may not be the dominant mechanism for sinusoidal GSH efflux (Ballatori and Rebbeor, 1998) since other proteins should be responsible (Ookhtens and Kaplowitz, 1998). The cloned canalicular GSH transporter (rcGSHt) (Yi et al., 1994) was also found to be an artifact, and had resulted in confusion over the mechanism of biliary GSH excretion (see Li et al., 1999b). But it is now clarified that the multidrug resistance-associated protein 2 (Mrp2) (Paulusma et al., 1996),
alternatively named the canalicular multispecific organic anion transporter (cMoat), is primarily responsible for biliary GSH output (Ballatori and Rebeor, 1998; Mittur et al., 1998) based on functional studies with cloned Mrp2 (Paulusma et al., 1999; Van Aubel et al., 1999) and with Mrp2 deficient rats (Fernandes-Checa et al., 1992). However, the relative contribution of Mrp2 over other unknown transport proteins in the overall biliary excretion of GSH remains unknown (Ballatori and Dutczak, 1994).

1.1.2 Glutathione Conjugation Reactions

A critical function of GSH is to serve as the co-substrate for conjugation with xenobiotics or endogenous substances. Being a soft nucleophile, the thiol group of GSH is capable of reacting spontaneously and enzymatically with compounds containing soft electrophilic carbon, nitrogen or sulfur centers.

Figure 1-1. Structure of GSH and conjugation with an electrophile (RX)

These reactions are catalyzed by several enzymes, including the cytosolic glutathione S-transferases (GSTs), microsomal GSTs (mGSTs), and leukotriene C4 (LTC4) synthase (see Table 1-1). The GSTs catalyze a variety of reactions such as 1) nucleophilic displacement from saturated or aromatic carbons, 2) Michael additions and 3) strained oxirane ring opening (see Table 1-2). However, non-conjugative functions such as isomerase and peroxidase activities have also been demonstrated for the GSTs.
Table 1-1. Summary of rat glutathione S-transferases (GSTs)

<table>
<thead>
<tr>
<th>Class</th>
<th>Sub-class</th>
<th>Other Known Names</th>
<th>Examples of Substrates(^a)</th>
<th>Tissues(^b)</th>
<th>% Total Hepatic GSTs and Zonation(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha (A)</td>
<td>1</td>
<td>Yα1, subunit 1a, ligandin</td>
<td>EA, CDNB, androstenedione</td>
<td>L, K, Lu, I</td>
<td>13% PV</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yα2, subunit 1b, ligandin</td>
<td>EA, CDNB, CuOOH</td>
<td>L, K, Lu, I</td>
<td>11% PV</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Yc1, subunit 2, GST AA</td>
<td>EA, CDNB</td>
<td>L, K</td>
<td>16%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Yk, subunit 8, GST K, Ya</td>
<td>EA, CDNB, 4-HNE</td>
<td>L, RBC, K</td>
<td>0.8%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Yc2, Yfetus, subunit 10</td>
<td>EA, CDNB, ADE</td>
<td>L</td>
<td>0.6%</td>
</tr>
<tr>
<td>Mu (M)</td>
<td>1</td>
<td>Yb1, subunit 3, GST A</td>
<td>EA, CDNB, 4-HNE, BSP, DCNB, STO</td>
<td>L, T, Lu</td>
<td>15% PV</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yb2, subunit 4, GST D</td>
<td>EA, CDNB, 4HNE, BSP, STO</td>
<td>L, T, B, I</td>
<td>26% PV</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Yb3, Yn1, Tb, subunit 6</td>
<td>EA, CDNB</td>
<td>T, B</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Yb4</td>
<td>CDNB</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Yn2, subunit 9</td>
<td>CDNB</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Yo, subunit 11</td>
<td>CDNB</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>Pi (P)</td>
<td>1</td>
<td>Yf, Yp, subunit 7, GST P</td>
<td>EA, CDNB, 4HNE, BPDE</td>
<td>K, Sk, Lu, B, I</td>
<td></td>
</tr>
<tr>
<td>Theta (T)</td>
<td>1</td>
<td>Subunit 5, GST E</td>
<td>DCM, CuOOH, EPNP</td>
<td>L</td>
<td>2.4% PV</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Yrs, subunit 12, GST M</td>
<td>EA, methylchloride</td>
<td>L, Lu</td>
<td>16% PV</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Subunit 13, mitochondrial GST</td>
<td>EA, CDNB</td>
<td>L</td>
<td></td>
</tr>
<tr>
<td>Zeta (Z)</td>
<td>1</td>
<td>MAA isomerase</td>
<td>DCA, MAA, CuOOH,</td>
<td>L, B</td>
<td></td>
</tr>
<tr>
<td>Microsomal</td>
<td>MGST-1</td>
<td>mgST</td>
<td>CDNB, 4HNE, haloalkenes, CuOOH, phospholipid hydroperoxides</td>
<td>L, St, K, B, T</td>
<td>PV</td>
</tr>
</tbody>
</table>

Adapted from Eaton and Bammler (1999), Hayes and Pulford (1995) and Commandeur et al. (1995).

\(^a\) EA, ethacrynic acid; CDNB, chlorodinitrobenzene; 4HNE, 4-hydroxynonenal; BPDE, benzopyrene-diol-epoxide; CuOOH, cumene hydroperoxide; DCA, dichloroacetate; EPNP, epoxynitrophenoxypropane; STO, styrene oxide; DCNB, dichloronitrobenzene; MAA, maleylacetoneacetate

\(^b\) L, liver; T, testis; Lu, lung; B, brain; K, kidney; Sk, skin; St, stomach; RBC, red blood cells; I, intestine

\(^c\) % total hepatic GST content excluding MGST-1 and Z1. PP, periportal; PV, perivenous.
Figure 1-2. Reactions catalyzed by GSTs

1. Nucleophilic displacement from an aromatic carbon

\[
\text{GSH} \quad \text{NO}_2\text{C}_{6}\text{H}_4\text{Cl} \quad \text{NO}_2\text{C}_{6}\text{H}_4\text{SG}
\]

1-chloro-2,4-dinitrobenzene (CDNB)

2. Nucleophilic displacement from a saturated carbon

\[
\text{GSH} \quad \text{Cl} \quad \text{C}=\text{O} \quad \text{Cl} \quad \text{Cl} \quad \text{Cl} \quad \text{C}=\text{O} \quad \text{GSH}
\]

Dichloroacetic acid (DCA)

3. Michael addition

\[
\text{GSH} \quad \text{OH} \quad \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}=\text{O} \quad \text{OH} \quad \text{CH}_2\text{CH}_2\text{CH}_2\text{CH}=\text{O} \quad \text{GSH}
\]

4-hydroxy-2-nonenal (4HNE)

4. Strained oxirane ring opening

\[
\text{GSH} \quad \text{HO} \quad \text{C}_{27}\text{H}_{18}\text{O} \quad \text{HO} \quad \text{C}_{27}\text{H}_{18}\text{O}
\]

Benzo[a]pyrene-7,8,9,10-oxide (BPDE)
5. Peroxidase activity

\[
\text{Cumene hydroperoxide (CuOOH)} + \text{GSSG} \rightarrow \text{phenol} + \text{H}_2\text{O} + \text{GSH}
\]

6. Isomerase activity

\[
\text{Androstene-3,7-dione} + \text{GSH} \rightarrow \text{compound}
\]

1.1.3 Glutathione S-transferases

The GSTs are members of a large superfamily of cytoplasmic enzymes with distinct and overlapping substrate specificities. The soluble, cytoplasmic enzymes are categorized into families or classes named Alpha (A), Mu (M), Pi (P), Theta (T) and Zeta (Z) (Hayes and Pulford, 1995) (Table 1-1). These exist as homo- or hetero-dimers among subunit proteins of a given class. In an attempt to minimize the confusion caused by different naming systems, a common nomenclature has been recently established. For example, rGST A1-2 identifies the rat (r) heterodimer composed of A1 and A2 subunits. By convention, subunits within a given class share at least 40% gene sequence homology, but many exceptions to this criteria exist. The tissue distributions of each subunit vary (Table 1-1) and the highest GST activities can be
found in the testis, liver, small intestine and kidneys (in descending activity). Within a given species, sex differences are observed in tissue isozyme distributions and total GST content. For instance, in the rat liver, total GST activity in male rats is greater than in females. Furthermore, male rat livers contain more Mu but less Alpha class enzymes than female rat liver. Species differences in subunit composition are also apparent as exemplified by the liver GST content. In humans, Alpha subunits constitute the majority of hepatic GSTs whereas Alpha and Mu classes predominate in the rat. Interestingly, the mouse liver contains mainly Pi class GSTs, a class which is absent in the native rat liver but is inducible by carcinogens (Satoh et al., 1985).

There is wide substrate selectivity among the GST classes and several important isozyme-specific probes have been used for functional assessments (Table 1-1). For instance, non-selenium dependent glutathione peroxidase activity is a feature of the Alpha subunits. The A4 subunit has a particular high activity towards alpha-beta unsaturated ketones such as 4-hydroxynonenal (4HNE). The Mu isozymes are most efficient in the conjugation of epoxides with GSH while the rGST M1 subunit appears to have the greatest substrate specificity for bromosulfophthalein (BSP). GST Pi subunits are active towards a variety of chemicals including epoxides and 1,4 unsaturated aldehydes. A specific Theta class enzyme (GST T1) is the isoform most responsible for the metabolism of dihaloalkanes (Sherratt et al., 1998) whereas GST Z1 catalyzes the GSH conjugation of α-haloalkanoic acids (Wempe et al., 1999). By contrast, many GSTs mediate the GSH conjugation of the classical substrates 1-chloro-2,4-dinitrobenzene (CDNB) and ethacrynic acid (EA) (Table 1-1).

1.1.5 Membrane-Associated Proteins in Eicosanoid and Glutathione Metabolism

Another superfamily of proteins involved in GSH conjugation reactions are termed the Membrane-Associated Proteins in Eicosanoid and Glutathione metabolism (MAPEG) (Jakobsson et al., 1999). As suggested by the name, these proteins are found in intracellular membranes and currently include the microsomal GSTs (e.g. MGST1) and LTC₄ synthase. The total number of enzymes in this superfamily is quickly increasing. In terms of substrate
specificity, MGST1 catalyzes the GSH conjugation of many substrates, including CDNB, and shows GSH-dependent peroxidase activity towards cumene hydroperoxide (CuOOH). Other members of this superfamily have greater substrate specificity consistent with their roles in eicosanoid synthesis and metabolism (Jakobsson et al., 1999).

1.1.6 Mechanism of GSTs

Each subunit of the cytosolic GST dimer contains binding sites for GSH (G-site) and substrate (H-site). Among most classes of GSTs, the G-site amino acid residues are well conserved (Dirr et al., 1994). Particularly important is a conserved tyrosine residue that is responsible for stabilizing the thiolate anion (GS') form of the co-substrate. GSTs lower the pKₐ of the bound GSH to ~6.0-6.6, or 2 pH units lower than GSH in solution, thus promoting nucleophilic attack of GSH on electrophiles (Armstrong, 1991, 1997). The H-site residues on GSTs are variable among classes, indicative of the broad substrate specificity of many isozymes. For the Mu and Pi classes, each subunit appears kinetically independent and non-interacting but some data suggest that the Alpha subunit activity may be influenced by the dimeric structure (Whalen and Boyer, 1998). Most kinetic investigations on GSTs have shown a random binding sequence for GSH and substrates in the mechanism of catalysis (Jakobson et al., 1979). However, based on the average isozyme dissociation constant for GSH (K_m^{GSH}) of 0.2-0.5 mM and the average cellular GSH concentration of ~5 mM, it is expected that GSH binds first, followed by the substrate (Armstrong, 1991). This is probably not the case for the Theta class subunits whose K_m^{GSH} is approximately 5 mM (Meyer, 1993). The higher affinity for GSH of the Alpha, Mu and Pi classes also contributes to the high affinity binding of product as compared with the substrate. For example, the K_m value for the GSH conjugate of CDNB (DNP-SG) for many rat isozymes is 50-100 times lower than that of the parent compound (Meyer, 1993), and product inhibition of the enzyme is likely to exist. This aspect of the GST-catalyzed mechanism has raised questions on the perceived role of the enzyme in detoxication (Meyer, 1993).
1.1.7 Role of GSH Conjugation in Xenobiotic Detoxication, Toxication and Carcinogenensis

Humans are continuously being exposed to many potentially toxic electrophiles from ingested foods, environmental sources and from drugs. GSH conjugation is generally considered a detoxication pathway because it provides protection against protein and DNA adduction by electrophilic xenobiotics (Hayes and Pulford, 1995). Through the process of GSH conjugation, the reactivity of a compound is diminished with increased hydrophilicity. Furthermore, GSH conjugation creates a molecular tag that can be recognized by cellular efflux transporters localized on the cell membrane (Hayes and Pulford, 1995). Once removed from cells, these conjugates can be efficiently excreted from the body via the bile or urine. There are a large number of cases illustrating the cytoprotective role of GSH conjugation, and the classic example is that for acetaminophen hepatotoxicity (Mitchell et al., 1973). Hepatic GSH depletion is required for the cytotoxicity of acetaminophen because increasing cellular GSH content by the administration of GSH-ethyl ester can abrogate toxicity (Puri and Meister, 1983). Presumably when cellular GSH becomes depleted, the reactive metabolite of acetaminophen cannot be detoxified by GSH conjugation and this electrophile is allowed to alter cellular homeostatic mechanisms.

By contrast, GSH conjugation can lead to the formation of direct and indirect toxic species. For example, haloalkenes (e.g. trichloroethene) readily form GSH conjugates (e.g. S-(1,2-dichlorovinyl)glutathione), and, following sequential metabolism, these can produce nephrotoxic metabolites (e.g. S-(1,2-dichlorovinyl)-L-cysteine) (Anders and Dekant, 1998). Similarly, polyphenolic-GSH conjugates formed from quinones have been shown to maintain the redox and electrophilic characteristics of their parent moieties (Monks and Lau, 1997). Cellular accumulation of these conjugates can lead to toxicity in susceptible organs (Monks and Lau, 1998).
GSH conjugation may also be an important factor in the susceptibility of humans to cancer. Genetic polymorphism occurs for some of the Mu, Theta and Pi isozymes, leading to non-functioning GST subunits or low activity variants. Several studies have shown an association between the hGSTM1, hGSTT1 and hGSTP polymorphisms with increased susceptibility to lung, bladder, brain, testicular and oral pharyngeal cancers (Eaton and Bammler, 1998). The most convincing evidence for a role of GSH conjugation in cancer susceptibility was demonstrated in the mGSTP knock-out mouse model (Henderson et al., 1998). GST Pi deficient mice were found to be more susceptible to the tumorigenic effects of a polycyclic aromatic hydrocarbon carcinogen in comparison to normal mice.

1.1.7 Role of GSH Conjugation in Drug Resistance

Intrinsic and acquired drug resistance is an important problem in cancer chemotherapy (Tew, 1994). It has been hypothesized that drug resistance in certain instances is the consequence of the high capacity of neoplastic tissues to detoxify antineoplastics via the GSH/GST pathway. This is suspected because levels of the Pi GSTs (and less commonly Alpha GSTs) are increased in most neoplasms (Tew, 1994), leading to greater metabolism of nitrogen mustard type alkylating agents such as chlorambucil (Dirven et al., 1996). In fact, clinical trials were attempted to modulate drug resistance with the use of GST inhibitors such as ethacrynic acid (O'Dwyer et al., 1991) and sulfosalazine (Gupta et al., 1995), or by the lowering tumour GSH with a GSH synthesis inhibitor (Calvert et al., 1998). Despite this level of attention, only weak resistance (Tew, 1994) or no clear association (Townsend et al., 1998) could be observed with studies on GSTs in vitro. Recently, it has been demonstrated that, in addition to GST up-regulation, cellular export of GSH conjugates may be an important factor which influences drug resistance. Townsend and colleagues have demonstrated that high expression of a GSH export pump (multidrug resistance-associated protein, MRP1) is synergistic with, or essential to, GST-mediated drug resistance towards some but not all alkylating agents (Morrow et al., 1998a; 1998b; 1998c; Diah et al., 1999). Similarly, evidence
exists towards the exudation of cisplatin-GSH conjugates from the cell by MRP2 as an important factor in drug resistance development for this alkylating agent (Cui et al., 1999). Taken together, these recent findings indicate the cytoprotective actions of GSH conjugation may only be realized when the GSH adduct is efficiently exported from the cell.

1.1.8 Role of GSH Conjugation in the Formation of Endogenous Substances

Endogenous GSH conjugates are cellular mediators (Wang and Ballatori, 1998). For example, leukotriene C₄ (LTC₄), which is produced from LTA₄ through the actions of microsomal LTC₄ synthase, is one of the mediators of the slow releasing substances of anaphylaxis. GSH conjugation regulates the levels of several prostaglandins (PGA₁, PGA₂, PGJ₂, 9-deoxy-Δ¹²-PGD₂, 9-deoxy-Δ⁷,Δ¹²-PGD₂) through activation and deactivation. Similarly, GSTs catalyze the formation of hepoxilin A₃-C from hepoxilin A₃, the former being implicated in having a role in neurotransmission. 4HNE is a product of the free-radical mediated lipid peroxidation of polyunsaturated fatty acids and is thought to be responsible for a variety of cellular responses including cytotoxicity, genotoxicity, enzyme inactivation, cell proliferation and gene regulation (Laurent et al., 1999). GSH conjugation of 4HNE is therefore regarded to have important regulatory and protective functions.

1.1.9 Hepatic GSH-Conjugate Export

As has been described in the previous sections, efficient export of GSH conjugates is an essential component of the cellular detoxication machinery. A lack of cellular efflux system for GSH conjugates would result in increased accumulation, leading to product inhibition of the GSTs and retention of the potentially toxic GSH conjugates. Because the liver is an organ with a high capacity for GSH conjugation, it follows that the organ possesses several systems for conjugate export. Usually, the majority of GSH conjugates formed within hepatocytes is excreted into bile while the remainder is transported into blood (Wahlander and Sies, 1981).
Several members of the multidrug-resistance associated protein (MRP) family that are expressed in liver are responsible for GSH conjugate export (Table 1-2). These ATP binding cassette (ABC) transporters were first discovered for their association with anthracycline resistance in a lung cancer cell line (Cole et al., 1991), but it is now apparent that these MRPs play important physiological functions in many organs, including the liver. MRP2 (Paulusma et al., 1996; Bückler et al., 1996; Ito et al., 1997), also termed the canalicular multispecific organic anion transporter (cMOAT), is responsible for the biliary excretion of GSH conjugates into bile as evidenced by direct functional assays of the cloned protein (Madon et al., 1997; Ito et al., 1998) and indirect in vivo studies with Mrp2 deficient mutant rats (TR', transport deficient Wistar rats; and EHBR, Eisai hyperbilirubinemic rats derived from Sprague-Dawley rats) (Oude Elferink et al., 1989; Kobayashi et al., 1990). An electrogenic transport mechanism has also been described for GSH conjugates with rat canalicular membrane vesicles (Ballatori and Truong, 1995) and this process has preference for low molecular weight species (< 400 kDa). The identity of the protein(s) responsible for this electrogenic transport is currently unknown.

One possible mechanism responsible for GSH conjugate efflux at the basolateral hepatocyte membrane is through the actions of MRP1. Like MRP2, MRP1 has been established as a transporter for GSH conjugates (Jedlitschky et al., 1996) and is found at low levels on the sinusoidal membrane of hepatocytes (Roelofsen et al., 1997). Recently, MRP3 was also localized on the basolateral membrane of human hepatocytes (Kool et al., 1999a) and was shown to transport the GSH conjugate of DCNB (DNP-SG). By contrast, Hirohashi et al. (1999) have found that rat Mrp3 (initially named MLP-2, Hirohashi et al., 1998) transported the GSH conjugates, DNP-SG and LTC4, poorly, but is better suited for glucuronide conjugates. Further functional characterization of MRP1 and MRP3 (and MRP6, Hirohashi et al., 1998; Kool et al., 1999b; Belinsky and Kruh, 1999) will be necessary to establish their relative contributions to GSH conjugate export at the sinusoidal membrane.
Table 1-2. Summary of Multidrug Resistance-Associated Proteins (MRPs)

<table>
<thead>
<tr>
<th>Name</th>
<th>Other Names</th>
<th>Substrates a (for all orthologs)</th>
<th>GSH Dependence b</th>
<th>Tissues c Hepatocyte Zonation &amp; Polarity d</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>MRP</td>
<td>GSH, GS-X (e.g. EA-SG), LTC₄, folate, MTX, Bilirubin-glucuronides, E₂₁βG, VCR, VP-16, AFB, CPT-11, SN-38, DNR, SLCT, Fluo-3</td>
<td>Yes</td>
<td>L, K, SI</td>
</tr>
<tr>
<td>MRP2</td>
<td>cMOAT</td>
<td>GSH, GSSG, GS-X (e.g. EA-SG), Bilirubin-glucuronides, LTC₄, Folate, MTX, protease inhibitors, E₂₁βG, pravastatin, temocaprilat, CPT-11, Fluo-3</td>
<td>Yes</td>
<td>L, K, SI</td>
</tr>
<tr>
<td>MRP4b</td>
<td>MOAT-B</td>
<td>Nucleoside analogs</td>
<td>?</td>
<td>Pr, Lu, H</td>
</tr>
<tr>
<td>MRP5b</td>
<td>MOAT-C PABC11SMRP</td>
<td>FDA, CMFDA</td>
<td>No?</td>
<td>H, B, M</td>
</tr>
<tr>
<td>MRP6</td>
<td>MOAT-E MLP-1ARA</td>
<td>Anthracyclines?</td>
<td>?</td>
<td>L, K, SI</td>
</tr>
</tbody>
</table>

a FDA, fluorescein diacetate; CMFDA, chloromethylfluorescein diacetate; E₂₁βG, estradiol-17β-glucuronide; MTX, methotrexate; VCR, vincristine; DNR, daunorubicin; SLCT, sulfotioholcholytaine; LTC₄, leukotriene C₄; AFB, aflatoxin B₁; E₃₀₄₀-G, E₃₀₄₀-glucuronide
b glutathione dependence for transport of some substrates (e.g. VCR)
c L. liver; K. kidney; B. brain; SI. small intestine; C. colon; A. adrenals; Pr. prostate; Pa. pancreas; H. heart; T. testes; Th. thyroid; Lu. lung; Sp. spleen; St. stomach; RBC. red blood cell
d PP, periportal; PV, perivenous; Kullak-Ublick, 1999
e Belinsky et al., 1998; McAleer et al., 1999
f Lee et al., 1998; Schuetz et al., 1999

The organic anion transporting polypeptide (oatpl) (Jacquemin et al., 1994) represents another transporter implicated in the transport of GSH conjugates from hepatocytes into blood. Although mainly associated with the uptake of organic anions (Meier et al., 1998) and cations (Van Montfoort et al., 1999), oatpl has been found to transport several GSH conjugates...
including LTC₄, DNP-SG and BSP-SG (Li et al., 1998; Pang et al., 1998). The driving force for solute uptake into hepatocytes by oatp1 appears to be the counter-transport of GSH down its concentration gradient (Li et al., 1998). In oatp1-transfected HeLa cells, transport of the classical substrate, bromosulfophthalein (BSP), was assumed to be bi-directional (Shi et al., 1995) since the investigators did not confirm the molecular species (BSP or BSP-SG) being transported out of the cells. Furthermore, studies with sinusoidal membrane vesicles indicated that GSH-conjugate transport could be inhibited by GSH (Kobayashi et al., 1990). Taken together, these observations suggest that oatp1 or other members of this transporter family, including oatp2 (Noé et al., 1997; Reichel et al., 1999), may be partially responsible for sinusoidal GSH conjugate efflux.

1.1.10 Hepatic GSH Conjugate Catabolism - The Intrahepatic Mercapturic Acid Pathway

Hepatocyte derived GSH conjugates undergo a series of catabolic steps which lead to the eventual formation of mercapturic acids (Figure 1-3). The initial step in this pathway is the export of the formed conjugate (X-SG) into blood or bile by mechanisms described above. This exposes GSH conjugates to the membrane-bound enzyme, γ-glutamyltranspeptidase (GGT), which catalyzes the γ-glutamyl bound cleavage in the formation of cysteinylglycine S-conjugates (X-CG). GGT is expressed on the canalicular and sinusoidal membranes of hepatocytes and the apical membranes of cholangiocytes (Lança and Israel, 1991). In rat, the majority of GGT is found on the canalicular membrane while high levels of enzyme are present on the sinusoidal membrane in hamsters and humans (Hinchman and Ballatori, 1994). Cysteinylglycine S-conjugates in the bloodstream can be efficiently taken up by hepatocytes (Jösch et al., 1998) or reabsorbed from the bile canaliculus (Hinchman and Ballatori, 1994) by mechanisms which are not well understood. Alternatively, X-CG may be further metabolized in the bile canaliculus by aminopeptidase M (APM) (Hirota et al., 1985) or dipeptidyl dipeptidase (DDP, or dehydropeptidase I, renal peptidase) (Kozak and Tate, 1982). Localized on the
hepatocyte canalicular membrane, these enzymes were thought to be responsible for cleavage of the glycyl residues in the formation of most cysteine S-conjugates (X-CYS). But recently, Jösch et al. (1998) demonstrated that most cysteinylglycine S-conjugate dipeptidase activity in liver was found not on the membranes but in the cytosol. Cysteine S-conjugates formed in the bile canaliculus are presumably transported into hepatocytes (Hinchman and Ballatori, 1994) to be further metabolized by microsomal cysteine S-conjugate N-acetylttransferase (mNAT) (Duffel and Jakoby, 1982) into mercapturates (N-acetylcysteine S-conjugates, X-NAC). This enzyme, requiring acetyl CoA as a co-substrate, is specific for cysteine S-conjugates and not for the typical substrates of cytosolic N-acetyltransferases (NAT). Mercapturates formed within the hepatocyte are readily excreted into bile or transported into the blood for excretion by the kidneys (Hinchman et al., 1998; Jösch et al., 1998).

1.1.11 Zonation of Hepatic GSH Conjugation

It is well established that hepatocytes within the liver acinus are phenotypically heterogeneous with respect to many biochemical functions (Jungermann, 1995). Spatial enzyme and protein gradients are commonly found among hepatocytes along the sinusoidal capillary beds beginning from regions nearest the blood inlet (periportal, PP or Zone 1) to those closest to the outlet (perivenous, PV, or Zone 3). It is therefore conceivable that the factors affecting hepatic GSH conjugation, in particular those pertaining to substrate uptake or in situ formation rate, GST concentration, GSH availability and GSH conjugate efflux capacity, may also be zonated.
Electrophiles (X) entering the liver or formed from a precursor (A) react with GSH spontaneously or enzymatically to form X-SG. Through several transport and metabolic steps, the GSH conjugate is converted to X-CG, X-CYS and X-NAC. See text for details.

1.1.11.1 Zonation of substrate uptake or in situ formation rate

Exposure of individual hepatocytes to substrates for GSH conjugation is determined by whether the electrophile is delivered to the liver from the blood as a preformed species or is metabolically formed from a precursor within the hepatocyte. For those substrates not requiring metabolic activation within hepatocytes, PP hepatocytes would be exposed to the highest solute concentration due to the direction of blood flow. Thus the capacity for upstream cells to eliminate the solute regulates the amount of electrophile encountered by PV hepatocytes.
In this regard, the membrane permeability of a solute also determines its distribution among hepatocytes within the acinus. For solutes that are highly lipophilic, a decreasing and exponential portal-to-central gradient in substrate concentration can be expected, and indeed, this has been shown for CDNB (Schön et al., 1988). However, for a large number of ionized solutes, carrier-mediated transport is required for hepatocyte uptake. In this instance, acinar heterogeneity in hepatocyte transport would be an important determinant in regulating the local rates of GSH conjugation.

With the use of functional and immunohistochemical methods, transporter heterogeneities have been identified (see reviews by Groothuis and Meijer, 1992; Gumucio and Guibert, 1993). For example, PV uptake has been described for cysteine (Saiki et al., 1992), glutamate (Burger et al., 1989; Tan et al., 1999) and α-ketoglutarate (Moseley et al., 1992) while glucose transporter 1 (Tal et al., 1990), rat organic cation transporter (OCT1) (Meyer-Wentrup et al., 1998), oatp2 (Kakyo et al., 1999; Reichel et al., 1999), and sodium-dependent dicarboxylate transporter (Chen et al., 1999) are more enriched in the centrilobular regions. This is in contrast to the sodium-dependent taurocholate transporting polypeptide (ntcp, Stieber et al., 1994) and oatp1 (Abu-Zahra et al., manuscript under review) whose acinar distributions are homogeneous. Periportal localization of the monocarboxylate transporter 1 (MCT1) has been suggested in a preliminary communication (Halestrap and Price, 1999). Therefore, there is the possibility that electrophiles which enter the liver from the bloodstream are preferentially transported into hepatocytes within a particular zone to result in regional GSH conjugation. However, this phenomenon has not been previously observed.

Zonal formation of electrophiles is a common occurrence due to the localization of oxidative metabolism by the cytochromes P450 (CYP) in liver. Most CYPs, with the exception of CYP2 isoforms, are found exclusively in the PV regions of the liver (see review by Oinonen and Lindros, 1998). Local activation of xenobiotics into electrophiles by PV hepatocytes has been implicated in centrilobular necrosis caused by bromobenzene (Reid and Krishna, 1973).
acetaminophen (Mitchell et al., 1973), carbon tetrachloride (Reynolds et al., 1975) and 1,1-dichloroethylene (DCE) (Forkert, 1999). In these examples, GSH conjugation of the reactive intermediates presumably occurs within the PV regions until co-substrate is depleted. Consequently, the balance between activation and detoxication is compromised and PV cell death ensues. The most convincing example of zonal GSH conjugation by in situ formed electrophiles is the case of DCE hepatotoxicity (Forkert, 1999). Using immunohistochemical techniques, Forkert demonstrated that CYP2E1-dependent formation of reactive DCE metabolites occurred in PV hepatocytes containing CYP2E1. In addition, immunodetectable GSH-DCE conjugates as well as covalently bound DCE were found to be colocalized within the same PV hepatocytes.

1.1.1.2 Zonation of GSTs

The concentration of the GSTs may limit the rates of GSH conjugation within the liver acinus particularly for substrates that are dependent on enzymatic conjugation rather than spontaneous conjugation. Redick et al. (1982) first studied the localization of GSTs by immunohistochemical methods and found that the staining of rGST A1-1, M1-2, and T1-1 was not uniform across the acinus, with a greater intensity found in the PV region. GST activity towards CDNB was also found to be greater in PV than PP hepatocytes in studies with microdissected tissues (El Mouelhi et al., 1986) and isolated, enriched PP and PV hepatocyte fractions (Kera et al., 1987; Bengtsson et al., 1987; Suolinna et al., 1989; Sippel et al., 1991; Gebhardt et al., 1994; Lindros et al., 1998). In these cases, the ratio of PV/PP activities was rather modest (~2), indicating a shallow and increasing (PP to PV) gradient in GSTs. High performance liquid chromatographic (HPLC) (Sippel et al., 1996) and immunohistochemical (Mainwaring et al., 1996; Otieno et al., 1997) methods have now shown that the majority of GST isozymes are found in greater abundance in the centrilobular region of the liver.
1.1.11.3 Zonation of GSH

A heterogeneous distribution of GSH was originally described by Smith et al., (1979) using a Prussian blue histochemical technique. These authors suggest that the concentration of GSH in the PP region is twice that of the PV zone. Similar findings were reported by others (Harisch and Meyer, 1985; Shimzu and Morita, 1990,1992) using the same method. However, these findings are in disagreement with other observations on GSH concentrations using o-phthalaldehyde (Murray et al., 1986) and mercury orange (Forkert and Moussa, 1989) histofluorescence, which indicate a lack of zonal heterogeneity. Similarly, GSH contents measured in isolated PP and PV hepatocytes were not different (Kera et al., 1988; Garcia-Ruiz et al., 1994, 1995). However, the extrapolation of the in vitro to the in vivo situation may not be appropriate because GSH levels are known to decrease during the cell isolation procedure. Recently, Fang et al. (1998) observed, using a dual-digitonin-pulse perfusion method, that hepatocytes in the PP and PV zones have similar GSH contents.

The rate of GSH synthesis within liver zones is an important factor determining both the steady-state GSH concentration and the susceptibility to continued chemical insult. With isolated PP and PV hepatocytes, Kera et al. (1988) demonstrated that the rate and eventual concentration of GSH was greater in PP than PV hepatocytes in the presence of precursor amino acids. Glutathione synthase activities were similar among the zonal hepatocytes but slightly higher activities of GCS were found in PV hepatocytes (Penttilä, 1990). Preferential uptake of cysteine by PV hepatocytes (Penttilä, 1990; Saiki et al., 1992), whereas homogeneous uptake of methionine, a precursor of cysteine (Penttilä, 1990), indicate that acinar efflux of GSH, the zonation of the transsulfuration pathway or the utilization of cysteine may explain the observation of higher GSH synthesis capacity of PP hepatocytes in comparison to PV hepatocytes.
1.1.11.4 Zonation of GSH conjugate efflux transporters

To date, the known transporters responsible for the biliary and sinusoidal efflux of GSH conjugates include the MRP and oatp families. Recently, Kool et al. (1999b) established that MRP2 was homogeneously localized in human liver. By contrast, MRP3 appeared to be localized only in PP hepatocytes (Kool et al., 1999b). The hepatic distributions of MRP1 and MRP6 have yet to be described. Rat oatp1 is distributed homogeneously in liver (Abu-Zahra et al., in review) whereas oatp2 has a PV distribution (Kakyo et al., 1999; Reichel et al., 1999). Thus far, there is no report comparing the functional efflux of GSH conjugates within PP and PV hepatocytes.

1.2 INTEGRATIVE VIEW OF GSH CONJUGATION IN WHOLE LIVER

In addition to the presence of GSH, and GSTs normally considered to be involved in the glutathione conjugation of acceptor substrates, the GSH conjugation in the intact liver is further affected by additional variables. These are:

1) blood flow rate through the liver,
2) transport of acceptor substrate between the vasculature and hepatocytes.
3) whether spontaneous or non-enzymatic conversion of the solute occurs.
4) binding of solutes to plasma proteins.

An index of the efficiency of solute removal by the liver is often denoted as the hepatic clearance (CL\textsubscript{h}), which is defined as the volume of incoming fluid cleared of solute per unit time. The values of clearance are therefore limited between \(0 \leq CL\textsubscript{h} \leq Q\), where \(Q\) is the blood flow rate to the liver. Another measure of hepatic drug elimination is the hepatic extraction ratio (E) which is defined by,

\[ E = \frac{CL\textsubscript{h}}{Q} \] (1-1)
and ranges in values between 0 (no removal) and 1 (complete removal).

1.2.1 Traditional Clearance Models

Several basic models of hepatic clearance that encompass the foregoing factors have been developed to describe and predict drug clearance (see review by Saville et al., 1992). These include the “well-stirred” (Rowland et al., 1973), “parallel-tube” (Winkler et al., 1973), “dispersion” (Roberts and Rowland, 1986) and “series compartment” (Weisiger, 1985; Gray and Tarn, 1987) models. For each of the basic hepatic clearance models, the solutions for steady-state extraction ratios ($E_{ss}$) can be obtained for linear kinetic conditions and under the assumption that solute binding to plasma proteins is in rapid equilibrium, that is, the fraction solute unbound in plasma ($f_u$) is constant along the sinusoids. The most important aspect which discriminates the models lies on the description of solute mixing within the vasculature. This ranges from complete mixing (“well-stirred” model) to no mixing (“parallel tube” model); the model predictions may be viewed as boundary conditions. For the “dispersion” and “series compartment” models, vascular mixing is intermediate between the extremes of the “well-stirred” and “parallel tube” models and are better approximations of the physiology of the liver. When a solute equilibrates rapidly between the blood and hepatocyte, as in the case for lipophilic, diffusible drugs, the equations for the steady state extraction ratio, $E_{ss}$, are much simplified (Pang and Rowland, 1977a; Roberts and Rowland, 1986; Gray and Tarn, 1987 and see equations to follow). For situations where these assumptions on membrane permeability do not hold true, the general forms for the equations describing $E_{ss}$ (Yamazaki et al., 1996) can be employed.

In the “well-stirred” model, the liver is represented as comprised of a rapidly mixed, hepatic blood and a cellular compartment. The concentration of solute is considered to be uniform throughout the blood compartment and equals to that of venous plasma. The general equation for $E_{ss}$ in the “well-stirred” model under first-order conditions is,
where $\text{CL}_{\text{int,all}}$ is defined as the overall intrinsic clearance (Yamazaki et al., 1996), which in actuality, is the "apparent" intrinsic clearance integrating solute influx and efflux permeabilities ($\text{PS}_{\text{in}}$ and $\text{PS}_{\text{out}}$, respectively) and removal activity (metabolic and excretory intrinsic clearance, $\text{CL}_{\text{int}}$).

\[
\text{CL}_{\text{int,all}} = \frac{\text{PS}_{\text{in}} \times \text{CL}_{\text{int}}}{\text{PS}_{\text{out}} + \text{CL}_{\text{int}}}
\]  

(1-3)

For the "parallel tube" model, substrate uptake occurs continuously along parallel sets of tubes, creating an exponential gradient in solute concentrations from the liver inlet to the outlet within each tube. In this scenario,

\[
E_{ss} = 1 - e^{-\frac{f_u \times \text{CL}_{\text{int,all}} \times Q}{f_u \times \text{CL}_{\text{int,all}} + Q}}
\]  

(1-4)

In the "dispersion model", blood flow through the sinusoids is non-ideal, reflecting the tortuosity of the hepatic vasculature. The degree of solute mixing within the sinusoids is parameterized with the dispersion number ($D_n$) whose value ranges between $\infty$ (complete mixing) and 0 (no mixing). On examining the dispersion of non-eliminated solutes traversing the liver, the most appropriate value for $D_n$ of ~0.22 has been obtained (Schwab et al., 1998). The equation for $E_{ss}$ for the dispersion model is given by,

\[
E_{ss} = 1 - e^{\frac{1 - \sqrt{1 + 4D_nR_n}}{2D_n}}
\]  

(1-5)

where $R_n$, or the efficiency number, is defined by,
In the "series compartment" model, the apparent mixing within the sinusoids results from movement of solutes along a sequence of $N$ numbers of well-stirred, vascular compartments, each with its respective cellular compartment. The degree of mixing is therefore determined by the number of serial compartments within the model. The model becomes the equivalent of the "well-stirred" and the "parallel tube" models when $N = 1$ and $\infty$, respectively. The function for $E_{ss}$ for the series compartment model is (Tam and Gray, 1987).

$$E_{ss} = 1 - (1 + \frac{f_u CL_{int,all}}{NQ})^{-N}$$  \hspace{1cm} (1-7)

These clearance models have been proven useful for predicting the hepatic clearances of drugs when perturbations in blood flow, metabolic clearance and protein binding are encountered (e.g. in particular disease states) (Pang and Rowland, 1977b; Morgan and Smallwood, 1990). Furthermore, they have been successfully applied for the scale-up of drug transport and metabolism data in vitro to in vivo (Yamazaki et al., 1996). However, the basic clearance models have some limitations in their description of hepatic drug removal. For example, solutions for $E_{ss}$ are readily obtained for the "parallel tube" and the "dispersion models" when kinetics are linear, but are poorer defined with the increasing nonlinearity for solutes undergoing saturable membrane transport or metabolism. Moreover, these models do not consider the effect of co-substrate availability for the non-linear, bimolecular reaction of solutes undergoing GSH conjugation. In addition, the model equations in their simplest forms do not take into account the possibility of non-uniform distribution of transport and metabolic systems within the liver acinus. Thus, for substrates metabolized by GSH conjugation, more complex approaches are necessary in order to adequately describe the hepatic clearance of acceptor substrates undergoing GSH conjugation.
1.2.2 Models That Incorporate Co-Substrate

Reports pertaining to toxicology are often interpreted quantitatively with the physiologically-based, pharmacokinetic modeling approach to describe the disposition of various chemicals and their conjugation with GSH. The majority of these studies employ the "well-stirred" model to describe the spontaneous, second-order reaction of electrophiles with GSH in liver (D'Souza et al., 1988; Chen and Gillette, 1988; El-Masri et al., 1996). In other instances, GSH conjugation reactions had been modeled using enzyme kinetic equations based on known catalysis by GSTs (Johanson and Filser, 1993; Csanády et al., 1994; Kedderis et al., 1996). GSH homeostasis was taken into account in most of the reported models through the inclusion of parameters describing GSH synthesis and natural turnover. In some examples, the induction of GSH synthesis after tissue depletion was also modeled (D'Souza et al., 1988; Frederick et al., 1992). With the extra consideration of co-substrate availability, these investigators were able to simulate the pharmacokinetic profile of administered chemicals and the tissue depletion of GSH. However, measurements and predictions of the disposition of the formed GSH conjugate that would have strengthened the validity of the models were not performed.

1.2.3 Models That Incorporate Zonation

With the understanding that the liver does not behave as a well-stirred compartment and that acinar drug transport and metabolism can influence hepatic drug elimination, several spatial kinetic models have been developed which incorporate such complexities. Pang and Stillwell (1983) developed the "enzyme-distributed, tubular flow model", a variation of the parallel tube model, to describe the elimination of highly permeable substrates by enzymes with variable acinar localizations. This model was used successfully to describe the elimination and metabolism of lidocaine (Pang et al., 1986), gentisamide (Morris et al., 1988) and salicylamide (Xu and Pang, 1989), during prograde and retrograde liver perfusions. Later, Miyauchi et al.
(1987) further extended the model to include a membrane permeability barrier for solutes and zonal metabolism, as did Kwon and Morris (1997) who included both zonal transport and elimination. The series compartment approach has been used to model the influence of enzyme zonation on drug elimination. Using a three compartments in series model with acinar and subcellular enzyme distributions, Tirona and Pang (1996) were able to describe the clearance and sequential metabolism of salicylamide.

1.2.4 Models Which Incorporate Zonation and Co-Substrate

For substrates undergoing hepatic GSH metabolism, the modeling of hepatic clearance requires inclusion of spatial (acinar) transport, metabolism and co-substrate availability. With this in mind, Chiba and Pang (1996) used the "enzyme-distributed tubular flow model" to describe the PV oxidation of acetaminophen to \( N \)-acetyl-\( p \)-quinone-imine (NAPQI) in order to predict zonal GSH depletion kinetics. The model fitted well with previously obtained data on acetaminophen metabolism in the perfused rat liver and inferred that perivenous GSH depletion would occur as a result of local drug bioactivation by the cytochrome P450s. However, many of the parameters used in their model were assigned or optimized due to lack of in vitro data, especially those regarding zonation, and the chosen parameters as well as specific zonal depletion of GSH were not validated. Nonetheless, this model provides an excellent foundation for further efforts to model hepatic GSH conjugation kinetics.

1.3 ETHACRYNIC ACID AS A MODEL COMPOUND TO STUDY THE KINETICS OF HEPATIC GSH CONJUGATION

1.3.1 Pharmacology

Ethacrynic acid ([2,3-dichloro-4-(2-methylene-1-oxobutyl) phenoxy] acetic acid); EA, Edecrin ®) was developed over 35 years ago for use as a diuretic (Figure 1-4). Originally, the diuretic effect of EA was believed to result from the reactivity of the \( \alpha,\beta \)-unsaturated ketone
moiety with renal protein sulfhydryls (see review by Koechel, 1981), but this was later dismissed with the discovery of several non-reactive EA analogs with diuretic activity. It has been suggested that the cysteine adduct of EA (EA-CYS) represents the active pharmacological species because it has strong diuretic action (Koechel and Cafruny, 1975). Furthermore, simultaneous infusion of cysteine with EA (to presumably promote CYS-adduct formation) induces a diuresis in rats, a species normally resistant to the effects of the drug (Kompanowska-Jezierska et al., 1990). The effect of EA is the result of inhibited Na+ and Cl− transport in the thick ascending limb of Henle (TALH) (Burg and Green, 1973), thus classifying EA as a loop diuretic. With the molecular cloning of the electroneutral Na-K-2Cl co-transporter (NKCC2) (Gamba et al., 1994), its functional inhibition by loop diuretics such as furosemide and bumetanide (Gamba et al., 1994) and its localization in the TALH (Obermüller et al., 1996), it is likely that the diuretic action of EA occurs through inhibition of NKCC2. Interestingly, EA-CYS was found to inhibit the functions of the closely related transporter, NKCC1, in erythrocytes with greater potency than EA (Palfrey and Leung, 1993). This result strongly suggests an important role for this EA metabolite in the overall pharmacologic effect.

1.3.2 Disposition In Vivo

The biological fate of EA was first described by Klaassen and Fitzgerald (1974) who found that, upon intravenous administration to rats and dogs, the majority of the dose could be found in bile as GSH conjugates or their sequential catabolites (Figure 1-4). Administration of EA to these species induced choleresis that was caused by the excretion of EA and its metabolites in bile. Furthermore, they found that the concentration of EA-GSH in bile was 45 to 450 times that of the plasma, suggesting active transport of the conjugate. Wallin et al. (1978) also showed that 80-90 % of the dose given intravenously to rats could be collected in bile mainly as EA-SG. Pretreatment with phenobarbital decreased the half life (t1/2) and increased the biliary excretion of EA-SG, an effect that was attributed to an induction of the GSTs. Analysis of the pharmacokinetic data, reveals the total body clearance of EA in rats is ~
3 ml/min or approximately 1/4 the liver blood flow rate. In addition, the result showed that only a minor percentage (1%) of the administered dose was covalently bound to liver tissue, with most of the radiolabelled protein co-eluting with the GSTs with gel filtration. In humans, EA was shown to be rapidly cleared from the blood, with a total body clearance ranging between 611 to 1405 ml/min (LaCreta et al., 1994). These values are similar to the blood flow rate to the liver in humans. Moreover, a low absolute bioavailability of < 21% was estimated. The cumulative data on EA disposition in different species suggest that the liver is responsible for most of the total body clearance of this drug and that hepatic GSH conjugation is the main elimination pathway.

1.3.3 Plasma Protein Binding

Ronwin and Zacchei (1967) first demonstrated that EA is bound avidly to bovine serum albumin (BSA) in vitro. They suggested that 4 moles of EA bind irreversibly and 12 mole of EA bind reversibly for each mole of protein. Later, Koechel et al. (1977) determined that EA reacts with the lone thiol of BSA and that the interaction was slowly reversible. Similarly, EA was found to bind strongly to the diazepam and the fatty acid binding sites on human serum albumin (HSA) (Fehske and Müller, 1986; Bertucci et al., 1999). There are currently no reports on the concentrations of plasma protein bound and unbound EA in vivo for any species.

1.3.4 Metabolism In Vitro

Habig et al. (1974) showed that GSH conjugation of EA was catalyzed by several rat GST isozymes. As more GSTs were isolated, it has become apparent that many rat (and

<table>
<thead>
<tr>
<th>Table 1-3.</th>
<th>K_m and V_max values for selected rGSTs towards EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>rGST</td>
<td>A1</td>
</tr>
<tr>
<td>K_m (μM)</td>
<td>90</td>
</tr>
<tr>
<td>V_max (μmol/min/mg)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Adapted from Ploemen et al. (1990, 1993)
human) GSTs are capable of metabolizing EA (Table 1-3). The specific activities of the GSTs towards EA varies, with rGSTA4 and rGSTP1 being the most efficient isoforms. Similarly, the affinities of the GSTs towards EA differs (Table 1-3).

EA undergoes conjugation with GSH nonenzymatically (Habig et al., 1974), and is rapid at physiological pH and GSH concentrations (Satoh, 1995). The chemical reaction of EA and GSH forms a product containing an asymmetric carbon (C9) (Fig. 1-4). Recently, it was demonstrated that the nonenzymatic GSH conjugation forms equal proportions of the two EA-SG diastereomers (van Iersel et al., 1998). However, with enzymatic catalysis by hGSTP1 and hGSTA1, one diastereomer of EA-SG is formed. This is not the case for hGSTA2 and rGSTM1 for which equal amounts of diastereomers are produced (van Iersel et al., 1998; Ploemen et al., 1993). Therefore, the proportions of EA-SG diastereomers formed in vivo would depend on the GST isozyme composition of particular cells and the degree to which spontaneous GSH conjugation occurs. This would be important if EA-SG efflux transporters, namely the MRPs, act stereospecifically. No other metabolites of EA have been described in the literature, however as with other GSH conjugates, EA-SG may be further catabolized to cysteinylglycine- (EA-CG), cysteine- (EA-CYS), and N-acetylcysteine- (EA-NAC) EA adducts (Klaassen and Fitzgerald, 1974) (Figure 1-4).

It is well recognized that EA is an inhibitor (and substrate) of GSTs (Ahokas et al., 1984). Importantly, EA-SG has been shown to be a more potent inhibitor of GSTs than EA itself (Ploemen et al., 1990, 1993a; Takamatsu and Inaba, 1992; Awasthi et al., 1993). As discussed previously, this phenomenon occurs for most substrate/conjugate pairs in their interactions with GST isozymes with the exception of the Theta class GSTs (Meyer, 1993). EA can covalently bind to the active site cysteine residue of rat and human GSTP1 (Ploemen et al., 1993b, 1994) to cause enzyme deactivation. At physiological GSH concentrations, this reaction is readily reversible indicating that covalent inhibition occurs only when cellular GSH is depleted.
Figure 1-4  Sequential Metabolism of EA

EA

\[
\begin{array}{c}
\text{HO-C-CH}_2-O-\text{C-C-CH}_2 \\
\text{Cl} \quad \text{Cl} \quad \text{CH}_2\text{CH}_3 \\
\text{ GST} \\
\end{array}
\]

EA-SG

\[
\begin{array}{c}
\text{HO-C-CH}_2-O-\text{C-C-CH}_2 \\
\text{Cl} \quad \text{Cl} \quad \text{CH}_2\text{CH}_3 \\
\text{H}_2\text{N} \quad \text{Am} \quad \text{NH} \quad \text{COOH} \\
\gamma\text{-GT} \\
\end{array}
\]

EA-CG

\[
\begin{array}{c}
\text{HO-C-CH}_2-O-\text{C-C-CH}_2 \\
\text{Cl} \quad \text{Cl} \quad \text{CH}_2\text{CH}_3 \\
\text{H}_2\text{N} \quad \text{SO} \quad \text{NH} \quad \text{COOH} \\
\text{DDP} \\
\end{array}
\]

EA-CYS

\[
\begin{array}{c}
\text{HO-C-CH}_2-O-\text{C-C-CH}_2 \\
\text{Cl} \quad \text{Cl} \quad \text{CH}_2\text{CH}_3 \\
\text{H}_2\text{N} \quad \text{SO} \quad \text{OH} \\
m\text{NAT} \\
\end{array}
\]

EA-NAC

\[
\begin{array}{c}
\text{HO-C-CH}_2-O-\text{C-C-CH}_2 \\
\text{Cl} \quad \text{Cl} \quad \text{CH}_2\text{CH}_3 \\
\text{NH} \quad \text{SO} \quad \text{OH} \\
\end{array}
\]
1.3.5 Transport of EA and EA Metabolites

The fact that EA is ionized at physiological pH but is efficiently eliminated by the liver through GSH conjugation indicates the potential presence of facilitative uptake mechanisms. By using the recirculating rat liver perfusion model and by measuring the initial disappearance of EA in the perfusate, Peterlik and Gazda (1980) deduced that the transport of EA into hepatocytes occurs by saturable, energy-dependent and partially sodium-dependent mechanisms. The saturable uptake system displayed a maximal uptake velocity of 0.64 μmol/min/g liver and a $K_m$ of 260 μM. Furthermore, the biliary excretion pattern of EA-SG indicated that this transport pathway was also saturable and exhibited a $V_{max}$ of 0.15 μmol/min/g liver. EA-SG was not detected in the perfusate, suggesting a lack of sinusoidal export by the hepatocyte. Other studies had examined the processes related to the renal excretion of GSH conjugates by measuring the transport of EA-SG in cultured primary rat proximal tubular cells (RPT) (Haenen et al., 1996). These investigators found that intact EA-SG was incapable of being transported from either the apical or basolateral poles of cultured RPT cells. Rather, catabolism of EA-SG to EA was first required for transport. To date, no specific protein has been identified with EA transporting capacity. However, the efflux of EA-SG out of cells has been shown to be mediated by MRP1 (Zaman et al., 1996) and MRP2 (Evers et al., 1998).

1.3.6 Toxicology

With appropriate clinical use, EA is a relatively safe drug with few side effects (Anonymous, 1999) and is considered one of the safest diuretics (Tolman et al., 1989). The most common side effects are related to the pharmacologic effect of the drug, i.e. electrolyte disturbance especially potassium, magnesium and calcium loss. Another side effect of EA common to other loop diuretics is ototoxicity which manifests as tinnitus, vertigo and in extremely rare cases, permanent deafness. The mechanism for this ototoxicity has recently been established by the use of NKCC1 knock-out mice lacking the Na-K-2Cl cotransporter (Delpire et al., 1999; Flagella et al., 1999). Absence of this transporter resulted in structural damage to
the inner ear, which caused profound deafness. This directly links the inhibition of NKCC1 in the ear by loop diuretics with their associated ototoxicities.

In vitro, EA has been shown to be cytotoxic to the hepatocyte at high concentrations (>200 μM) with evidence of disrupted membrane integrity beginning after 1-2 hour of exposure (Meredith and Reed, 1982; Dogterom and Mulder, 1993) while others have shown the lack of cytotoxicity of 500 μM EA over a 2-hour incubation (Silva and O'Brien, 1989). High concentrations (800 μM) of EA produced overt cytotoxicity to isolated rat hepatocytes within 30 min (Khan and O'Brien, 1991). With primary rat hepatocyte cultures, a 6-18 hour exposure to EA produced a concentration-dependent cytotoxicity (Tolman et al., 1989; Anderson et al., 1998) with a median toxic concentration of 33 μM.

The cytotoxic mechanism of EA was studied in cultured cerebellar astrocytes in which an increased level of reactive oxygen species was observed after an initial depletion of GSH (Huang and Philbert, 1996). This was later followed by a decline in mitochondrial membrane potential and eventually plasma membrane disruption. Similar results were shown in cultured rat cerebellar granule neurons (Wüllner et al., 1999). These findings agree with those demonstrating the inhibition of mitochondrial respiration by EA (Manuel et al., 1976; Seyfried et al., 1999) and EA-induced changes to mitochondrial morphology (Soltys and Gupta, 1994). Together, these results indicate that disruption of mitochondrial function by EA is an important factor involved in the cytotoxic mechanism of this drug. Other cellular process may have been altered because EA is known to affect the functions of a variety of enzymes including Na+/K+ ATPase (Banerjee et al., 1971), glutathione reductase (Kurata et al., 1992; Hoffman et al., 1995; Huang and Philbert, 1996), DT diaphorase (Joseph et al., 1994) and glyceraldehyde-3-phosphate dehydrogenase (Huang and Philbert, 1996).
1.3.7  EA as a Model Drug for the Study of Hepatic GSH Conjugation

The key features that make EA a good substrate to study the factors influencing hepatic GSH conjugation are:

1) EA is solely eliminated by GSH conjugation because other metabolic pathways are unknown,
2) previous studies have suggested that the rat liver efficiently metabolizes EA.
3) rat hepatic GSTs are known to mediate the enzymatic conjugation of EA with GSH.
4) hepatic EA uptake may occur by facilitative transport, thus allowing for the study of the interplay between drug uptake and metabolism, and
5) EA is relatively non-toxic among electrophiles at low concentrations and for short exposures.
CHAPTER 2

STATEMENT OF PURPOSE OF INVESTIGATION
The liver is the most important xenobiotic biotransformation organ of the body and plays a central role in regulating levels of drugs and metabolites and thus their associated activities and toxicities. Glutathione (GSH) conjugation is a detoxification reaction performed by the liver that renders electrophiles, including drugs, environmental chemicals and endogenous substances, less reactive and more hydrophilic such that they can be readily excreted by the liver and/or the kidneys. However, in some instances, GSH conjugation is an activating step in the toxification of xenobiotics. Given the pharmacologic and toxicologic significance of this metabolic pathway, prediction of the GSH conjugation capacity of the liver towards particular substrates would therefore have practical implications.

For substances undergoing hepatic GSH conjugation, the overall organ clearance is determined by the complex interplay of several factors - hepatic blood flow, plasma protein binding, membrane permeability and transport, enzymatic activity, propensity for spontaneous reactions and co-substrate (GSH) availability. In addition, the efficiency of conjugate efflux is a determinant of clearance since GSH conjugates are product inhibitors of the enzyme, glutathione S-transferase (GST). These interactions occur in a distributed-in-space fashion among hepatocytes situated along the sinusoidal flow path. Because phenotypic differences exist among hepatocytes within the liver acinus, in particular the centrilobular enrichment of GSTs, it becomes essential to consider spatial aspects for each of these clearance factors since they can further modulate local rates of GSH conjugation and the overall efficiency of hepatic drug removal.

A concerted approach must be taken to integrate the in vitro factors to in vivo. Given the complex interrelationships existing among membrane permeability, enzymatic activity, co-substrate availability and the possibility of inherent acinar heterogeneities, each of these factors must be viewed simultaneously to predict hepatic GSH conjugation rates of acceptor substrates. The best approach for the comprehensive description and prediction of hepatic GSH conjugation is through an examination of all the processes and their assemblage into a distributed-in-space scheme.
2.1 Hypotheses

1. *In vitro* metabolic data on GSH conjugation, when scaled-up, reflect the overall metabolic activity for the whole liver.

2. *In vitro* transport data from isolated rat hepatocytes, when scaled-up, reflect the uptake and excretion in the whole liver.

3. Kinetic analyses of the individual processes and their zonation are necessary to predict local GSH conjugation between the inlet and outlet of the liver in a distributed-in-space fashion.

2.2 Objectives

1. To determine the rate-limiting steps involved in the GSH conjugation of EA in perfused rat liver by studying
   (a) spontaneous (non-enzymatic) and rat liver cytosol-enhanced (enzymatic) GSH conjugation kinetics of the model compound, ethacrynic acid (EA), *in vitro*
   (b) the transport kinetics of EA into isolated rat hepatocytes

2. To assess possible acinar heterogeneities of the variables affecting the GSH conjugation of EA using enriched, isolated periportal and perivenous rat hepatocytes

3. To integrate the composite *in vitro* data to predict the rates of hepatic GSH conjugation of EA that occur between the inlet and outlet of the liver in a distributed-in-space fashion.
CHAPTER 3

BIMOLECULAR GLUTATHIONE CONJUGATION KINETICS OF ETHACRYNIC ACID IN RAT LIVER: IN VITRO AND PERFUSION STUDIES

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3.1 ABSTRACT

The conjugation kinetics of glutathione (GSH) and ethacrynic acid (EA) were studied in rat liver perfusion studies, where efficient removal occurred (steady state extraction ratio $E_s$, ~0.4 - 0.8 at concentrations ranging from 10 - 200 μM) despite the appreciable plasma protein binding. The declining $E_s$ paralleled the saturation in glutathione conjugate (EA-SG) formation: EA-SG primarily appeared in bile as the unchanged glutathionyl adduct (90%) and minimally as cleavage products. The GSH conjugation of EA in perfused liver was described by the constants $K_m^{\text{overall}}$ of 67 μM and $V_{\text{max}}^{\text{overall}}$ of 0.23 μmol/min/g liver. These differed from those observed for the bimolecular, nonenzymatic (constant of 126 μM$^{-1}$ min$^{-1}$) and enzymatic ($K_m$ for GSH and EA were 1.2 mM and 94 μM, respectively; $V_{\text{max}}$ of 533 nmol/min/mg liver cytosolic protein or 32 μmol/min/g liver) GSH conjugation of EA in vitro. But they were similar to those estimated for EA uptake in isolated rat hepatocytes by saturable ($K_m^{\text{uptake}} = 57$ μM, and $V_{\text{max}}^{\text{uptake}} = 0.55$ μmol/min/g liver) and non-saturable (0.015 ml/min/mg) processes. At increasing EA concentrations (>25 μM), time-dependent changes were further observed for $E_s$ and EA-SG formation, which rapidly decreased with time after the attainment of steady state due to the rapid loss of cellular GSH. The composite data were described adequately by a physiological model that accounted for transport and the glutathione-dependent conjugation of EA. The results suggest that the rate-limiting process for hepatic EA glutathione conjugation is cellular uptake, but co-substrate availability controls the rate of metabolism when GSH becomes depleted.

3.2 INTRODUCTION

The metabolism and biliary excretion of the loop diuretic, ethacrynic acid (EA), was described over two decades ago by Klaassen and Fitzgerald (1974) who demonstrated that EA was rapidly cleared from the plasma of rats and dogs, with the majority of the dose being excreted in bile as the glutathione (GSH) conjugate (EA-SG). Wallin et al. (1978) also observed a high clearance of EA with a rapid plasma half-life ($t_{1/2}$) of 19 min in the rat, and the value could be decreased further significantly with phenobarbital pretreatment. Later, LaCreta et al. (1994)
described the pharmacokinetics of EA in cancer patients and established that the oral bioavailability of the drug was less than 21% due primarily to a high first-pass effect. Taken together, these studies suggest that the liver is the major organ involved in the total body clearance of EA.

The glutathione S-transferases (GSTs) that comprise about 4% of total cytosolic protein in liver are responsible for the high capacity, metabolic inactivation of electrophilic compounds. Indeed, EA was been shown to be a substrate of several constitutively expressed rat liver GST isozymes including the α (subunits 1,2 and 8) and μ (subunits 3 and 4) classes (Habig et al., 1974. Ploemen et al., 1990), although nonenzymatic GSH conjugation also exists. Furthermore, it was shown that EA-SG was an inhibitor of the GSTs due to its greater affinity for the enzymes than that of the parent molecule (Ploemen et al., 1990) , whereas EA itself inhibits GST π through reversible covalent interactions (Ploemen et al., 1994). Because of these properties and because of the ability of EA to deplete cellular GSH, there is the potential clinical usefulness of EA as a modulator of drug resistance linked with the GSH/GST system (O’Dwyer et al., 1991).

There are only sparse examples in the literature where the factors affecting the kinetics of hepatic GSH conjugation of xenobiotics are examined experimentally and integrated into predictive models. Thus, the purpose of this study was to study the bimolecular, GSH conjugation kinetics of the model drug, EA, in rat liver with the goal of understanding the interplay between the factors affecting protein binding, hepatic transport, cosubstrate availability, and enzymatic/non-enzymatic conversion. For this purpose, EA conjugates were synthesized, and a stable HPLC assay was developed for their quantification. Non-enzymatic/enzymatic GSH conjugation was examined in vitro. Cellular uptake by isolated rat hepatocytes was studied to describe transport, inasmuch as the influx clearances of various drugs in the intact liver have been found to correlate closely to those found in isolated rat hepatocytes (Yamada et al., 1997). Protein binding, conjugate formation, and cellular GSH were examined at increasing EA concentrations to the perfused rat liver to ultimately describe the concentration-dependent hepatic GSH conjugation of EA. We were able to show that the in vitro factors affecting hepatic EA removal could be successfully scaled-up to describe the bimolecular reaction of glutathione conjugation at the organ level with use of a
physiologic model that included synthesis and degradation of the cofactor GSH. Our results suggest that cellular uptake rate-limited GSH conjugation of EA in rat livers perfused with erythrocyte-free media. When hepatic GSH was depleted, co-substrate availability became the rate-limiting step in EA elimination.

3.3 MATERIALS AND METHODS

3.3.1 Chemicals. EA, cysteine, cysteinylglucose, N-acetylcysteine (NAC), 4-(2,4-dichlorophenoxy)-butyric acid (DCPBA), GSH, GSSG, γ-glutamyl-glutamate, bathophenanthroline-disulfonic acid, m-cresol purple, 2,4-dinitrofluorobenzene, iodoacetic acid, taurocholate (TCA), bromosulfophthalein (BSP), bumetanide (BUM), estradiol-17β-glucuronide (E2-17β-G), and bovine serum albumin (25% solution in Tyrode’s buffer) were obtained from Sigma Chemical Co. (St. Louis, MO). HPLC-grade acetonitrile was purchased from Caledon Laboratories, Mississauga, ON, Canada). [^{14}C]EA (specific activity 15 mCi/mm mol) was a gift from Dr. J.H.T.M. Ploemen (TNO, Zeist, The Netherlands) and this was further purified by HPLC and solid phase extraction (radiochemical purity >98%). [^{3}H]Sucrose (specific activity 10 Ci/mm mol) was obtained from New England Nuclear (Boston, MA). All other regents were of analytical grade.

3.3.2 Synthesis of EA-Thiol Conjugates. EA cysteinyl adducts - EA-CYS and EA-CG - were synthesized by the method of Klaassen et al. (1974) and according to Cavrini et al. (1987) for EA-SG and EA-NAC, with minor modifications. EA (0.1 to 1 g) in 50 ml of ethanol was combined with equimolar amounts of L-cysteine or cysteinylglucose (in 50 ml of water) or GSH or NAC (in 50 ml of 10 mM potassium phosphate, pH 7.0) for 30 to 60 min. After drying under reduced pressure, the crude products were purified initially by preparative TLC (silica gel GF, 1000 μm: Analtech, Newark, DE) with a solvent system of acetonitrile:water (7:2 vol/vol) as described by Awasthi et al. (1993). The Rf values were 0.41, 0.36, 0.24, 0.43, 0.82 for EA-CYS, EA-CG, EA-SG, EA-NAC and EA, respectively. EA-CYS and EA-CG were further purified by gel filtration (Sephadex G-10, Pharmacia, Uppsala, Sweden) on a 4 x 12 cm column eluted with water at a flow
rate of ~ 2 ml/min. The eluent was collected and analyzed by HPLC, and the fractions eluting at 45 - 65 min and 25 - 50 min were pooled for the EA-CYS and EA-CG fractions, respectively, and lyophilized. EA-SG was purified by solid phase extraction using Sep-Pak C_{18} cartridges (2 g) (Waters, Milford, MA). The EA-SG loaded cartridges were washed with 4 ml of water followed by 12 ml of 20% CH_{3}CN, and the conjugate was eluted with 50% CH_{3}CN. EA-NAC was purified by acid extraction (pH~1 with 1N HCl) into ethyl acetate (2 x 25 ml). Identification of the final products was carried out by mass spectrometry (Sciex API III, MDS-Sciex, Thornhill, Ontario, Canada) that gave protonated molecular ions [MH^+] m/z of 424, 481, 610 and 466 for EA-CYS, EA-CG, EA-SG and EA-NAC, respectively (Figs. 3-1 and 3-2). Analysis of the EA-thiol conjugates by the HPLC method described below indicated chemical purities > 99% for EA-CYS and EA-NAC, >98% for EA-CG, and >97% for EA-SG.

3.3.3 Stability of EA-Thiol Adducts. The stability of EA and the EA-thiol conjugates at pH 7.4 and 3.2 was examined at 37°C. These pH conditions were chosen for study because they represented those encountered physiologically and during the assay procedure, respectively. Solutions containing 1 mg EA or each of the EA-thiol conjugates dissolved in 10 ml of 100 mM potassium phosphate were incubated at 37°C. An aliquot of 200 µl was removed at various times, mixed with 20 µl of 1N HCl, 50 µl of internal standard (4.1 mM DCPBA in CH_{3}CN) and subjected to HPLC analysis as described below. EA was stable at both pH conditions (7.4 and 3.2) for the duration of the incubations (Figs. 3-3A and 3-3C). All of the thiol metabolites were stable at pH 3.2 (Fig. 3-3C), but were unstable at pH 7.4 (Fig. 3-3A) with decay half-lives of 0.32, 0.76, 8.5 and 59 h for EA-CYS, EA-CG, EA-SG and EA-NAC, respectively. Retro-Michael cleavage that results in formation of the parent compound has been suggested as the mechanism for degradation of these metabolites (Haenen et al., 1997), because the decline in the thiol conjugates was associated with the appearance of EA (Fig. 3-3B). For this reason, all perfusate and bile samples were acidified immediately upon collection prior to storage or analysis.
Figure 3-1  Mass spectrometric analysis of chemically synthesized EA-SG and EA-CG.
Figure 3-2  Mass spectrometric analysis of chemically synthesized EA-CYS and EA-NAC.
3.3.4 HPLC Assay for EA and Its Thiol Conjugates. For the assay of EA and metabolites in perfusate plasma, standard curves were prepared by adding various amounts of EA (2 to 200 μM) and its conjugates (2 to 100 μM) to acidified blank plasma (0.1 ml 1N HCl with 1 ml blank plasma). After the addition of 50 μl of internal standard (4.1 mM DCPBA in CH₃CN) (LaCreta et al., 1991) and an additional 100 μl of 1N HCl, 6 ml of CH₃CN was added for protein precipitation. Preliminary attempts to precipitate the proteins with methanol resulted in the degradation of EA and formation of a new product, presumably the methyl ester described by Midha et al. (1978). Upon centrifugation at 3000xg for 10 min, the supernatant was removed and dried under N₂ gas at ambient temperature. The resulting residue was re-constituted with 200 μl HPLC diluent [mobile phase A : mobile phase B (65:35) as described below], and a portion of this was injected into the liquid chromatograph. Plasma samples were processed in an identical fashion. Bile was diluted 200 to 400 fold with the HPLC solvent, spiked with 165 nmol of DCPBA, then injected into the HPLC. The species were quantified with use of calibration curves consisting of EA and its conjugates (1 to 100 μM) and the internal standard.

The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of two pumps (LC-6A), a UV detector (SPD-6A) set at 275 nm, an auto-injector (SIL-6A), an integrator (C-R3A), and a system controller (SCL-6A). A binary gradient consisting of mobile phase A (50 mM potassium phosphate, pH 3.2) and mobile phase B (CH₃CN) was utilized at a constant flow rate of 1 ml/min for the separation of EA and the thiol conjugates by a reversed-phase column (μBondapak C₁₈, 3.9 x 300 mm, Waters, Milford, MA). Initially, the mobile phase was maintained at 18% B for 25 min followed by a linear increase to 35% B by 30 min, and was maintained at 35% B for another 15 min before being brought back down to 18% B over the next 5 min. The mobile phase was kept at 18% B for another 5 min to allow for column re-equilibration. Calibration curves were constructed with the area ratio method. For validation of the assay procedures, standard curves for EA and its thiol adducts prepared in perfusate (plasma) were assayed on three separate days. Intra-day variability of the assay was determined in triplicate perfusate plasma samples spiked with high (50 to 100 μM) and low (2 to 10 μM) concentrations of EA and its metabolites with HPLC.
Figure 3-3. Stability of EA and thiol metabolites in (A) 100 mM potassium phosphate buffer pH 7.4 and (C) 3.2 at 37°C. Formation of EA from thiol metabolites at pH 7.4 (B).

3.3.5 Nonenzymatic and Enzymatic (Rat Liver Cytosol-Catalyzed) EA-SG Formation. The spontaneous rate of EA-SG formation in absence of GST was first determined at various concentrations of EA (25 to 200 μM) and GSH (1 to 10 mM) by the method of Habig et al.
(1974) as described by Satoh (1995). Briefly, reactions were carried out in 100 mM potassium phosphate, pH 7.2, at 37°C and were initiated by the addition of 300 μl of EA dissolved in 40% ethanol to GSH (3 ml final reaction volume). The UV absorbance at 270 nm was determined at 10 to 20 sec intervals using a Uvicon 860 spectrophotometer (Kontron Instruments, Everett, MA) until the completion of the reaction (5 to 8 min) (Fig. 3-4). The pseudo first-order rate constant, $k_1$, was calculated from a plot of log (absorbance at $\infty$ - absorbance at time t) versus time, and upon multiplication of this value by the initial concentration of EA, furnished the reaction rate ($v$) as determined with the equation, $k_1 [\text{EA}]_{\text{initial}} = v$. The calculated $v$ was in turn used to estimate $k_2$, the second-order rate constant, by a least squares minimization procedure (SCIENTIST, MicroMath Scientific Software, Salt Lake City, UT) according to the equation, $v = k_2 [\text{GSH}]_{\text{initial}} [\text{EA}]_{\text{initial}}$ with data generated from different concentrations of EA and GSH.

For examination of the influence of hepatic enzymes (GST) on the rate of GSH conjugation of EA, rat liver cytosol (100,000 g supernatant, ~0.1 mg protein/ml) was included in the reaction mixture which contained various concentrations of EA (25 to 200 μM) and GSH (0.25 to 5 mM). The reaction rates were normalized to the concentration of protein that was quantified by the method of Lowry et al. (1951) with bovine serum albumin (BSA) used as the standard. After correction for the spontaneous reaction, the enzymatic rates were fitted to a single enzyme, rapid equilibrium random sequential scheme as proposed by Jacobson et al. (1979):

$$v = \frac{v_{\text{max}}^{\text{metab}} [\text{EA}][\text{GSH}]}{K_m^{\text{GSH}} K_m^{\text{EA}} + K_m^{\text{EA}} [\text{GSH}] + K_m^{\text{GSH}} [\text{EA}] + [\text{EA}][\text{GSH}]}$$

(3-1)

where $K_m^{\text{GSH}}$ and $K_m^{\text{EA}}$ are the apparent Michaelis-Menten constants and $v_{\text{max}}^{\text{metab}}$, the maximal conjugation rate of the bimolecular reaction, and [EA] and [GSH] denote concentrations.

### 3.3.6 Uptake of EA by Isolated Hepatocytes.

Hepatocytes from male Sprague-Dawley rats (295 to 315 g) were prepared as described by Hassen et al. (1996). These were suspended in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with glucose (5 mM) and HEPES (1 mM). Their viability exceeded 90% as assessed by trypan blue exclusion. In the first set of
uptake studies, concentration-dependency for EA uptake was investigated by the addition of a mixture of \[^{3}H\]sucrose (extracellular marker), \[^{14}C\]EA, and varying amounts of unlabeled EA in

**Figure 3-4** Consecutive UV spectral analysis of spontaneous EA-SG formation with 200 \(\mu\)M EA, 5 mM GSH, pH 7.2 at 37°C. UV scans at 12 sec intervals.

0.9% NaCl to achieve final EA concentrations of 1 to 800 \(\mu\)M in the hepatocyte suspension (\(-1.67 \times 10^{6}\) cells/ml). At 15 to 20 sec intervals, samples (50 \(\mu\)l) of the incubation mixture were removed and immediately filtered through silicon oil into NaOH (bottom layer) and analyzed for cellular uptake; aliquots (25 \(\mu\)l) of the supernatant were taken for radioactivity counting. In *cis*-inhibition experiments, the uptake of \[^{14}C\]EA (25 \(\mu\)M) was studied with or without TCA (200 \(\mu\)M), BSP (20 \(\mu\)M), E\(_{2}\)-17\(\beta\)-G (10 \(\mu\)M), and bumetanide, BUM (200 \(\mu\)M) at inhibitor concentrations that were saturating for hepatocyte uptake (Meier *et al.*, 1997). Na\(^{+}\)-dependency for EA uptake was investigated in experiments whereby the sodium chloride and sodium bicarbonate in the incubation medium were replaced by choline chloride and potassium bicarbonate, respectively. The \[^{14}C\]EA taken up into hepatocytes was analyzed by liquid scintillation spectrometry (Model LS5801, Beckman Canada, Mississauga, ON), after correcting for the adhered extracellular water layer defined by \[^{3}H\]sucrose. The uptake rates of EA in the presence of inhibitors and in absence of sodium ion were expressed as percentages of the control uptake rates.
Because the accumulation of EA into hepatocytes was linear over 60 to 80 sec, regression of the accumulation profile yielded a slope that designated the initial uptake velocity ($v_{\text{uptake}}$). For experiments in which varying EA concentrations were used, the $v_{\text{uptake}}$ was fitted versus the substrate concentration [EA] according to Eq. 3-2 with a least-squares fitting routine (weighting scheme of $1/[\text{EA}]$).

$$v_{\text{uptake}} = \frac{v_{\text{max}} ^{\text{uptake}} [\text{EA}]}{K_m^{\text{uptake}} + [\text{EA}]} + P_{\text{diff}} [\text{EA}]$$

(3-2)

where $K_m^{\text{uptake}}$ and $v_{\text{max}} ^{\text{uptake}}$ denote, respectively, the Michaelis-Menten constant and the maximal uptake rate for the saturable component, and $P_{\text{diff}}$ is the uptake clearance for the linear component. To estimate whether or not passive diffusion is a contributing factor in cellular uptake, the partitioning of [14C]EA between n-octanol and 100 mM potassium phosphate buffer (pH 7.4) was studied. Equal volumes of buffer and n-octanol containing [14C]EA were vigorously mixed in a separatory funnel. The two phases were allowed to separate overnight and the radioactivity in the octanol and buffer was determined by scintillation spectrometry. An equilibrium value of 3.1 (octanol BUFFER) was found.

The uptake clearance ($PS_{\text{in}}$ or influx rate/concentration) at the organ level could be estimated by the following equation (Sasabe et al., 1997):

$$PS_{\text{in}} = \left( \frac{v_{\text{max}} ^{\text{uptake}}}{K_m^{\text{uptake}} + [\text{EA}]} + P_{\text{diff}} \right) \left( \frac{\alpha}{\beta} \right)$$

(3-3)

and the maximal influx clearance is given by

$$PS_{\text{in}, \text{max}} = \left( \frac{v_{\text{max}} ^{\text{uptake}}}{K_m^{\text{uptake}} + P_{\text{diff}}} \right) \left( \frac{\alpha}{\beta} \right)$$

(3-3A)

where $\alpha$ is $1.25 \times 10^6$ cells/g liver and $\beta$ is $1 \times 10^6$ cells/mg protein.

3.3.7 Protein Binding of EA to BSA in Perfusate. The extents to which various concentrations of EA (2 to 400 μM) bind to albumin (1%) in perfusate, pH 7.4, was studied by equilibrium dialysis at 37°C (Spectrapor, molecular cutoff at 12,000 to 14,000 Da; Spectrum
Medical Industries Inc., Los Angeles, CA). Preliminary experiments indicated that equilibrium was achieved within 2 h, and dialysis was carried out over 3 h. The protein and buffer compartments were analyzed by HPLC.

The binding data were best fitted to the following binding isotherm which includes one saturable and one non-saturable binding component:

\[
C_p = \frac{n[P]C_u}{K_D + C_u} + k_u C_u + C_u
\]  

(3-4)

where \(C_p\) and \(C_u\) represent the total and unbound concentrations of EA in plasma, respectively, \(n\) is the number of binding sites, \(K_D\) is the dissociation constant for the saturable binding site, \([P]\) is the BSA concentration, and \(k_u\) is the non-saturable proportionality binding constant.

3.3.8 Erythrocyte-Free Rat Liver Perfusion. Red blood cell metabolism of EA to EA-SG was noted in preliminary liver perfusion studies conducted with 20% red blood cells (Fig. 3-5). Hence an erythrocyte-free perfused liver preparation was used to avoid the added complication. For adequate oxygenation of the liver, perfusate plasma (1% albumin and 300 mg% glucose in Krebs Henseleit bicarbonate solution, pH 7.4) was delivered at a flow rate of 25 ml/min to the livers of male Sprague-Dawley rats (241-340 g; liver weight, 8.2 - 13.6 g obtained from Charles River, St. Constant, QC). Surgery was performed under pentobarbital anesthesia (50 mg/kg, intraperitoneally), and the surgical procedure and perfusion apparatus were identical to those described previously (Pang et al., 1988). Each liver was perfused through the portal vein in absence of an arterial contribution, and the perfusate exited via the hepatic vein; the hepatic artery was ligated. Bile was collected through a cannula (PE10 tubing) inserted into the bile duct. The temperature of the liver was maintained at 37°C with a heat lamp. Livers were perfused for 120 min with drug-free medium in order to assess the viability of livers undergoing erythrocyte-free perfusion. Liver viability was assessed by gross appearance, bile flow rate, oxygen consumption
Figure 3-5  GSH conjugation of (A) ~400 µM EA and (B) ~80 µM EA by perfusate containing bovine RBCs (20% v/v). (C) Total suspension GSH content in control and EA treated RBCs.
monitored by a Clark-style oxygen electrode, Instech Laboratories, Horsham, PA) and leakage of alanine aminotransferase (ALT), assayed by a commercially available colorimetric kit obtained from Sigma.

3.3.9 EA Liver Perfusion Experiments. In EA perfusion experiments, livers were initially perfused for 10 min with drug-free perfusate for equilibration of the liver preparation, followed by a 90 min-perfusion with EA-containing medium of only one concentration (8.2 to 194 μM). At 0, 30, 60 and 90 min, the input perfusate reservoir was sampled for the determination of EA concentration. The mean of these determinations was used to denote the steady state input concentration, C_{in,ss}. At 5, 10, 15, 30, 45, 60, 75 and 90 min, the outflow perfusate was collected for determination of EA (C_{out}) and metabolite concentrations; the mean of the determinations that had remained constant and maximal was taken as C_{out,ss}, the steady state output concentration. For perfusate collection, 0.1 ml of 1 N HCl was added to 1 ml of perfusate immediately after sampling in order to avoid degradation of the metabolites. Bile was allowed to drain directly into tared glass vials containing 50 μl of 1 N HCl at 10 min intervals. The difference in weight before and after bile collection was taken to be the volume of bile, after taking the assumption that the density of bile is 1.0 g/ml. The acidified plasma and bile samples were stored at -20°C until analysis. At the completion of each experiment (90 min), the liver was quickly weighed and a section was immediately processed for total GSH and GSSG content using the derivatization and HPLC method of Fariss and Reed (1987).

The steady-state extraction ratio (E_{ss}) of EA was estimated from the relation that E_{ss} = (C_{in,ss} - C_{out,ss})/C_{in,ss}, and was the mean of 6 to 8 determinations. However, at high EA concentrations (Experiments #1 to #4), E_{ss} was estimated as the mean of the determinations obtained between 15 and 30 min, because values of E beyond these times declined with time. The total amount of EA or EA-SG appearing in the venous outflow plasma or bile was estimated as the area under the plasma concentration-time curve or area under the excretion rate-time curve from t = 0 to 90 min for each species, with use of the linear trapezoidal rule. Recovery was determined by
summing the total plasma and biliary output of EA and its metabolites and expressing this value as a fraction of the total amount of EA administered.

3.3.10 Physiological Modeling. The disposition of EA was modeled with use of a physiological model which assumes well-stirred liver plasma and cellular compartments as shown in Figure 3-6 (see the appendix for mass balance equations and parameters obtained in vitro) (see later in Table 3-2). The saturable uptake \( K_m^{\text{uptake}} \) and \( V_{\text{max}}^{\text{uptake}} \) and the bidirectional (uptake and efflux) linear clearance \( P_{\text{diff}} \) parameters were scaled up from isolated hepatocyte uptake data (Eqs. 3-3 and 3-3A) to describe the sinusoidal EA transmembrane movement. Modeling efforts included consideration of plasma protein binding, and the unbound EA traverses across membranes and becomes eliminated. Because the apparent Michaelis-Menten constants for GSH \( K_m^{\text{GSH}} \) and EA \( K_m^{\text{EA}} \) were known from in vitro cytosol-catalyzed EA metabolism experiments, only the maximal conjugation rate \( V_{\text{max}}^{\text{metab}} \) was estimated by the fitting procedure. Because all EA-conjugates (EA-SG, EA-CG, and EA-CYS) in bile originate from EA-SG or from intrabiliary hydrolysis (Hinchman et al., 1991), all metabolite species, including the EA-NAC that arose from EA-CYS were summed to provide an estimate of the biliary excreted EA-SG. The EA-SG formed in the liver cell was assumed to undergo net sinusoidal efflux, denoted by the first-order clearance, \( P_{\text{diff}}(\text{EA-SG}) \), and undergoes biliary excretion with the biliary intrinsic clearance denoted by \( CL_b(\text{EA-SG}) \); these parameters were fitted. The intracellular GSH content was governed by a zero-order synthesis rate (of rate constant \( K_{\text{syn}}(\text{GSH}) \)); the consumption of GSH via conjugation with EA is again a bimolecular reaction whereas the degradation accounting for its natural turnover is a first-order removal process of clearance, \( CL_{\text{deg}}(\text{GSH}) \). These constants were retrieved from the literature (Johanson and Filser, 1993; Chen and Gillette, 1988) while the initial cellular GSH concentration \( [GSH_c^{\text{ini}}(\text{GSH})] \) that may vary among livers was obtained through fitting, because the parameter is a key determinant of GSH conjugation. The fitted parameter values, together with the assigned parameters, are shown in Table 3-2. Data from individual experiments (plasma and bile output rates of EA and conjugates, and the GSH content in liver) were simultaneously fitted to the model (Fig. 3-6) with use of a nonlinear least squares procedure (Scientist) and a weighting
scheme of 1/observed value. All output rates were adjusted according to the recoveries of individual experiments.

**Figure 3-6** Scheme for the hepatic disposition of EA and its glutathione conjugate, EA-SG in the physiological kinetic model. EA uptake is by saturable (denoted by \( V_{\text{max}}^{\text{uptake}} \) and \( K_m^{\text{uptake}} \)) and nonsaturable (\( P_{\text{diff}} \)) processes. Glutathione conjugation of EA proceeds enzymatically catalyzed by the glutathione S-transferases (GSTs, with kinetic constants \( K_m^{EA} \), \( K_m^{GSH} \), and \( V_{\text{max}}^{\text{metab}} \)) and nonenzymatically (with the bimolecular constant, \( k_2 \)). The intracellular concentration of GSH was maintained by its synthesis (\( k_{\text{syn}}^{\text{GSH}} \)) and degradation (\( CL_{\text{deg}}^{\text{GSH}} \)). The EA-SG formed undergoes biliary excretion (\( CL_{\text{b}}^{\text{EA-SG}} \)), or undergoes net efflux (\( P_{\text{diff}}^{\text{EA-SG}} \)). See text for details of the model.

![Scheme for the hepatic disposition of EA and its glutathione conjugate, EA-SG](image)

### 3.3.11 Data Calculations

Data were presented as mean±S.D. For comparisons, ANOVA was performed and a \( p \) value of 0.05 was viewed as significant.
3.4 RESULTS

3.4.1 HPLC Assay. With the EA conjugates being acid-stabilized during sample collection, the new HPLC system was capable of separating EA and its thiol metabolites in biological fluids. The retention times were 16, 20, 23, 35, 40 and 43 min for EA-CYS, EA-CG, EA-SG, EA-NAC, EA and the internal standard, DCPBA, respectively (Fig. 3-7). The standard curves for EA and the conjugates in plasma and bile were linear ($r^2 > 0.99$) over the concentrations studied. The inter-day coefficients of variation for EA and its metabolites ranged from 2 to 8%, whereas intra-day variation ranged from 2 to 11%.

**Figure 3-7** Separation of EA-CYS, EA-CG, EA-SG, EA-NAC, EA and DCPBA (internal standard) by HPLC (retention times of 14.3, 16.8, 19.3, 35.0, 39.4, 42.4 min, respectively).

3.4.2 Nonenzymatic and Enzymatic EA-SG Formation. The spontaneous conjugation of EA with GSH was rapid at pH 7.2 (Fig. 3-8A), and the rate was linearly related to the concentrations
of EA and GSH. The second order rate constant, $k_2$, for nonenzymatic GSH conjugation was estimated to be $126 \pm 4 \mu M^{-1} min^{-1}$ (mean $\pm$ SD of estimate) with computer fitting ($r^2 = 0.978$). Addition of rat liver cytosol to EA (25 to 200 $\mu M$) and GSH (0.25 to 5 $mM$) resulted in increased rates of GSH conjugation above and beyond the spontaneous rate (Fig. 3-8B). Fitting of the data to Eq. 3-1 furnished estimates of the dissociation rate constants for EA ($K_m^{EA} = 94 \pm 16 \mu M$) and GSH ($K_m^{GSH} = 1.2 \pm 0.2 mM$), and for the $V_{max}$ of the reaction ($533 \pm 49 nmol/min/mg$ protein or $32 \mu mol/min/g$ liver based on 60 mg of cytosolic protein/g liver). The observations confirm that under physiological conditions, the spontaneous GSH conjugation rate of EA is quite high, as found by Satoh (1995), but the enzyme-catalyzed GSH conjugation rate of EA in the rat liver cytosol greatly exceeds the spontaneous rate, as also observed by Ploemen et al. (1993).

3.4.3 Uptake by Isolated Hepatocytes. EA uptake was linear over the 60 to 80 sec (Fig 3-9) and displayed concentration-dependency (Fig. 3-10). Fitting of $V_{uptake}$ (initial velocity obtained from the slope of the accumulation-time curve) vs. EA concentration (Eq. 3-2) furnished the kinetic constants for uptake: $K_m^{uptake}$ of $57 \pm 22 \mu M$, $V_{max}^{uptake}$ of $4.4 \pm 1.8 nmol/min/mg$ or $0.55 \mu mol/min/g$ liver, and $P_{diff}$ of $0.015 \pm 0.006 ml/min/mg$ (mean $\pm$ SD). Upon scaling of the cellular uptake parameters, the maximal influx clearance of EA at the organ level was estimated to be $11.4 ml/min/g$ liver (Eq. 3-3A), a value that is about 5 times the value of perfusate flow to the liver. EA (25 $\mu M$) uptake was further found to be sodium-independent, and was $cis$- inhibited by anions BSP, TCA and BUM (25 to 50% inhibition) but not by $E_2-17\beta-G$ (Table 3-1).

Table 3-1. Effect of Na$^+$ replacement and organic anions on $[^4C]$EA (25 $\mu M$) uptake by isolated rat hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>EA Uptake Rate (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$^+$ → Choline</td>
<td>92 ± 16</td>
</tr>
<tr>
<td>$E_2-17\beta-G$ (10 $\mu M$)</td>
<td>93 ± 6</td>
</tr>
<tr>
<td>TCA (200 $\mu M$)</td>
<td>77 ± 4 *</td>
</tr>
<tr>
<td>BSP (10 $\mu M$)</td>
<td>70 ± 7 *</td>
</tr>
<tr>
<td>BUM (200 $\mu M$)</td>
<td>51 ± 9 *</td>
</tr>
</tbody>
</table>

* Statistically significant from control rate ($p<0.05$) using Student’s t-test
(n=3-4). Control uptake rate was $2.0 \pm 0.3 nmol/min/10^6$ cells.
Figure 3-8  (A) Nonenzymatic (spontaneous) and (B) enzymatic (rat liver cytosolic protein enhanced) EA-SG formation in 100 mM potassium phosphate buffer pH 7.2 at 37°C and initial EA and GSH concentrations of 25-200 μM and 0.25-5 mM respectively (n=3 rat livers). Surface plot obtained by nonlinear regression analysis to equations found in the text.
Figure 3-9  Time course of EA uptake by isolated rat hepatocytes (n=4). Lines obtained from linear regression.
Figure 3-10  Concentration-dependent uptake of EA by isolated rat hepatocytes (n=4). The initial velocity was obtained from the slope of the accumulation vs. time data. The fitted line (—) from nonlinear least-squares fitting to Eq. 3-2 consisted of a saturable (---) and non-saturable (—) component.

3.4.4 Protein Binding of EA to 1% BSA in Perfusate. EA exhibited tight binding to perfusate plasma consisting of 1% BSA. The unbound plasma fraction ($f_u$) ranged from 0.02 to 0.07 (Fig. 3-11B) over the concentrations studied (2 to 400 μM). Results arising from non-linear fitting of data to Eq. 3-4 suggest the presence of saturable and non-saturable binding, yielding $1.3 \pm 0.3$ binding sites (or n) of $K_D$ (dissociation constant) of $2.6 \pm 1.1$ μM, and a linear binding constant, $k_L$ of $6.9 \pm 1.9$ (fitted value ± SD of the estimate) (Fig. 3-11A).

3.4.5 Liver Viability. Rat liver perfusion conducted with EA at concentrations up to 200 μM (n=10) showed a lack of observable deleterious effects towards the liver viability and leakage of aminotransferase was minimal (Fig. 3-12A). Oxygen consumption was maintained at an adequate level (> 2 μmol/min/g) for the entire 90 min of perfusion (Fig. 3-12B), and the values were generally similar to those for control livers (n=3) as well as other erythrocyte-containing perfusion
systems (Pang et al., 1988). Bile flow rates were rather constant (about 1 µl/min/g) in control livers (data not shown), but were increased linearly in relation to EA-SG excretion (Fig. 3-12C), showing that an additional 20 µl of bile flow was associated with approximately 1 µmol of EA-SG excreted (Fig. 3-12C).

**Figure 3-11** Protein binding of EA to perfusate containing 1% BSA. (A) Plot of the total ($C_t$) and unbound ($C_u$) plasma concentrations and (B) unbound fraction vs. the total plasma concentration as determined by equilibrium dialysis at 37°C. The line was obtained by fitting of the data to Eq. 3-4. The line is the fitted line based on the fitted parameters.
Figure 3-12  Characterization of the viability of the erythrocyte-free rat liver perfused at 25 ml/min by (A) aminotransferase activity in venous perfusate and (B) oxygen consumption. The bile flow rate of the perfused liver was further presented as a function of EA-SG excretion rate (C) [n=3 for control (○); n=10 for EA (*)].
3.4.6 Concentration-dependent Metabolism of EA in Perfused Rat Liver. Essentially all of the input dose was recovered as EA or its thiol metabolites in outflow perfusate and bile (recovery 101±8%), confirming that GSH conjugation is the major metabolic fate of EA in rat liver (Table 3-3). Biliary excretion of unmetabolized EA was insignificant (0.2% of biliary species). The majority of the species in bile existed mainly as EA-SG, with lesser proportions as the cleavage metabolites (EA-CG>EA-CYS>EA-NAC). EA-SG was the only metabolite found in outflow perfusate and represented 11 ± 6% of all metabolites escaping the liver. Steady-state was achieved within 10 to 20 min after EA perfusion. At low EA concentrations (< 50 μM), steady-state removal was maintained throughout the perfusion period, whereas at higher concentrations, removal was constant only for an initial, short attainment of steady-state and declined thereafter (> 30 min perfusion) (Fig. 3-13A); the same was observed for EA-SG formation (Fig. 3-13B), which was maximal between 15 to 30 min of perfusion. Lack of maintenance of steady-state at high EA concentration was likely due to depletion of GSH. Indeed, the intrahepatic GSH and GSSG contents in several of the liver preparations at the end of perfusion were low and were inversely related to the input EA concentration, denoting that depletion of GSH by EA was also concentration-dependent (Fig. 3-14).

The extraction ratio of EA existing at the steady state (E<sub>ss</sub>) between 15 to 30 min decreased from a high value of ~0.8 to 0.4 (Fig. 3-15A), whereas the corresponding steady-state formation rates of EA-SG (sum of all metabolites escaping into bile and outflow perfusate) displayed saturation with increasing EA input concentration (Fig. 3-15B). When the EA-SG formation rate was fitted against the logarithmic average EA concentration, \( \hat{C} = [(C_{\text{in,ss}} - C_{\text{out,ss}}) / \ln(C_{\text{in,ss}} / C_{\text{out,ss}})] \) as an estimate of the substrate concentration in liver with the simple Michaelis-Menten equation, \( v_{\text{renal}} = (V_{\text{max}}^{\text{overall}} \times \hat{C}) / (K_m^{\text{overall}} + \hat{C}) \), values of 67 ± 46 μM for \( K_m^{\text{overall}} \) and 0.23±0.7 μmol/min/g liver for \( V_{\text{max}}^{\text{overall}} \) (fitted value ± SD of estimate) were obtained.
Figure 3-13  Concentration- and time-dependent GSH conjugation of EA in the single pass perfused rat liver. (A) The output rates of EA in venous perfusate, (B) biliary excretion rates of EA-SG (sum of all thiol metabolites), and (C) GSH concentration in liver were plotted vs. time. Each symbol represents the results of an individual experiment with a single EA input concentration (10 to 200 μM), and the lines were obtained through least-squares fitting with the physiological kinetic model shown in Fig. 2-6.
Figure 3-14 Hepatic GSH (■) and GSSG (○) contents remaining in liver after 90 min of perfusion with various EA input concentrations.

Figure 3-15 The steady-state kinetics of removal of EA during single pass rat liver perfusion at various EA input concentrations: (A) the extraction ratio of EA and (B) overall EA-SG formation rate (sum of all thiol metabolites) (B) were presented. The line was obtained by least-squares fitting to the Michaelis-Menten equation.
3.4.7 Physiologic Modeling. Several strategies were tried for physiological modeling with use of the mass transfer equations shown in the appendix. Because the bimolecular kinetics on in vitro cytosol-catalyzed (enzymatic) glutathione conjugation of EA were consistent with a single enzyme described by the rapid-equilibrium random sequential scheme (Eq. 3-1), a similar equation that included both the enzyme-mediated and spontaneous glutathione conjugation was used to describe the GSH conjugation rate of EA in the perfused liver (see Appendix). The uptake parameters ($K_{m}^{uptake}$, $V_{max}^{uptake}$, and $P_{diff}$) obtained from isolated hepatocyte studies were used in the fitting procedure.

An approach that included fast-dissociation of the protein-bound EA (rapid equilibration between bound and unbound species) was first utilized to preserve the notion that the unbound species participated in transfer and removal. The predicted $E_{eq}$'s and associated removal rates were, however, too low in comparison to those observed. The use of the $K_{m}^{uptake}$ and $V_{max}^{uptake}$ and $P_{diff}$ in combination with other parameters, failed to predict the high hepatic extraction of EA unless all of the EA in perfusate, bound and free, were treated as if they were free species. Hence, the assumption that all EA, bound and unbound, were able to participate in mass transfer was taken and the equations describing the mass transfer of EA and EA-SG in the appendix implied the total concentration. Under this instance, the fit of the hepatic venous EA and biliary EA-SG output rates of each experiment to the physiologic model (Fig. 3-6) was satisfactory (Fig. 3-13), although the fit of the hepatic venous EA-SG output data was generally poorer; the latter might be due to the oversimplification of the transmembrane transport process of EA-SG ($P_{diff}(EA-SG)$) as a linear, unidirectional process. The underlying mechanism for EA-SG transport, however, would not impact on the estimation of the kinetic constants for formation of EA-SG.

The fitted parameters are summarized in Table 3-2. Estimates of the initial cellular GSH concentration yielded a mean value of 8.0 ± 2.0 mM, a value which is consistent with average rat hepatic GSH content (~ 6 μmol/g liver or ~ 9 mM given 0.64 ml cell volume per g liver) in control livers perfused with drug-free perfusate. Although there was much variability in the initial GSH concentration among livers (Fig. 3-13C), it was necessary to individually fit this parameter
because the use of a common value for all livers resulted in poorer fits. The estimate of \( K_m^{GSH} \) (1.2 mM) was of the same order of magnitude as others found previously (0.2 to 0.5 \( \mu \)mol/g or 0.3 to 0.8 mM) (Polhuijs et al., 1992; Snel et al., 1995). The value of the fitted \( V_{max}^{metab} \) (3.2 \( \mu \)mol/min/liver) was much lower than that estimated from \textit{in vitro} experiments (320 \( \mu \)mol/min for a 10 g liver) and the reason was not apparent. The discrepancy existing between the \textit{in vitro} and fitted \( V_{max}^{metab} \) is likely due to the poorer estimation of this parameter at the low EA input concentrations because saturation in metabolism was not approached under this condition.

3.5 DISCUSSION

In the present study, we confirmed that GSH conjugation of EA occurred spontaneously, but the rate was considerably less than that for enzyme-catalyzed GSH conjugation (Fig. 3-8). Cellular uptake studies with isolated rat hepatocytes revealed that EA uptake was concentration-dependent and could be described by saturable and linear processes (Fig. 3-10). At low concentrations (< 25 \( \mu \)M) and those seen clinically (LaCreta et al., 1994), hepatic uptake of EA is mediated mainly by a carrier-mediated process that lacks sodium-dependence and shows observable but modest inhibition by TCA, BSP and \( E_2-17\beta-G \) (Table 3-1), high affinity substrates/inhibitors of oatp1 (Meier et al., 1997). The data suggests that EA is not a substrate of ntcp, the sodium-dependent taurocholate transporter or oatp1, because > 86% inhibition was calculated for TCA, BSP and \( E_2-17\beta-G \) with competitive inhibition. The demonstrable inhibition by bumetanide, another loop diuretic that is transported by a third yet unknown anion transporter distinct from oatp1 and ntcp (Horz et al., 1996), opens the possibility that the transporter(s) responsible for EA uptake may be similar to that for bumetanide. The sodium-independence for EA uptake in isolated hepatocytes is inconsistent with reduced uptake of EA in the sodium-free recirculating liver perfusion model (Peterlik et al., 1980); the difference might be due to indirect effects of sodium-deprivation on liver viability. The appreciable, linear uptake transport clearance (\( P_{air} \)) of 19 ml/min for a 10 g liver may be attributed to the lipophilicity of EA, inasmuch as the n-octanol-buffer partitioning ratio was 3.1 at pH 7.4, implying concentration of EA in lipid membranes.
Table 3-2  Parameters for the physiological kinetic model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q</td>
<td>Perfusate flow rate</td>
<td>25 ml/min&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>$V_p$</td>
<td>Volume of sinusoid compartment</td>
<td>0.4 ml/g liver&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$V_c$</td>
<td>Volume of cellular compartment</td>
<td>0.64 ml/g liver&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>$v_{\text{max}}^\text{uptake}$</td>
<td>Maximum facilitated uptake velocity of EA</td>
<td>5.47 μmol/min/liver&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_m^\text{uptake}$</td>
<td>Michaelis-Menten constant for EA uptake</td>
<td>57 μM&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>$P_{\text{diff}}$</td>
<td>Bi-directional sinusoidal transmembrane clearance for EA</td>
<td>19 ml/min&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$v_{\text{max}}^\text{merub}$</td>
<td>Maximum glutathione conjugation rate</td>
<td>11.6 ± 6.7 μmol/min/liver&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_m^\text{EA}$</td>
<td>Michaelis-Menten constant for EA in bimolecular enzymatic reaction</td>
<td>94 μM&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_m^\text{GSH}$</td>
<td>Michaelis-Menten constant for GSH in bimolecular enzymatic reaction</td>
<td>1.2 mM&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Spontaneous GSH conjugation rate constant</td>
<td>126 μM&lt;sup&gt;f&lt;/sup&gt;·min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>$K_{\text{syn}}$</td>
<td>Zero-order GSH synthesis rate</td>
<td>0.1 μmol/min/liver&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{\text{deg}}(\text{GSH})$</td>
<td>GSH degradation clearance</td>
<td>0.003 min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{\text{S}}(\text{EA-SG})$</td>
<td>Sinusoidal transmembrane clearance of EA-SG</td>
<td>0.11 ± 0.06 ml/min&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>$CL_{\text{b}}(\text{EA-SG})$</td>
<td>Biliary intrinsic clearance of EA-SG</td>
<td>0.99 ± 0.29 ml/min&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>$GSH_{\text{c}}^{\text{10}}$</td>
<td>Initial cellular GSH concentration</td>
<td>8.3 ± 2.3 mM&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> condition for perfusion  
<sup>b</sup> obtained from Pang et al., 1988  
<sup>c</sup> from hepatocyte uptake experiments and scaled up using Eq. 3-3A  
<sup>d</sup> from hepatocyte uptake experiments  
<sup>e</sup> from rat in vitro liver cytosol-enhanced EA metabolism experiments  
<sup>f</sup> fitted using physiological model  
<sup>g</sup> from Johanson and Filser, 1993  
<sup>h</sup> from Chen and Gillette, 1988
Table 3-3  Summary of dose recovery during rat liver perfusion.

<table>
<thead>
<tr>
<th>Study</th>
<th>Liver Weight (g)</th>
<th>C&lt;sub&gt;in&lt;/sub&gt; (µM)</th>
<th>Biliary Output</th>
<th>Hepatic Venous Output</th>
<th>Total Metabolism</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>% of Input Rate</td>
<td>% of Total bile</td>
<td>% of Input Rate</td>
<td>% of Perfusate</td>
</tr>
<tr>
<td>1</td>
<td>9.2</td>
<td>8</td>
<td>78</td>
<td>88.3</td>
<td>11.7</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>13.6</td>
<td>12</td>
<td>75</td>
<td>97.0</td>
<td>3.0</td>
<td>0.0</td>
</tr>
<tr>
<td>3</td>
<td>12.0</td>
<td>15</td>
<td>70</td>
<td>92.0</td>
<td>8.0</td>
<td>0.0</td>
</tr>
<tr>
<td>4</td>
<td>8.4</td>
<td>28</td>
<td>44</td>
<td>93.0</td>
<td>6.7</td>
<td>0.0</td>
</tr>
<tr>
<td>5</td>
<td>9.6</td>
<td>49</td>
<td>53</td>
<td>94.7</td>
<td>5.0</td>
<td>0.1</td>
</tr>
<tr>
<td>6</td>
<td>10.2</td>
<td>93</td>
<td>30</td>
<td>94.9</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>7</td>
<td>11.4</td>
<td>112</td>
<td>19</td>
<td>99.2</td>
<td>0.6</td>
<td>0.0</td>
</tr>
<tr>
<td>8</td>
<td>8.6</td>
<td>152</td>
<td>14</td>
<td>96.5</td>
<td>1.7</td>
<td>0.6</td>
</tr>
<tr>
<td>9</td>
<td>11.0</td>
<td>153</td>
<td>16</td>
<td>98.5</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>10</td>
<td>8.2</td>
<td>194</td>
<td>14</td>
<td>94.1</td>
<td>4.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Mean</td>
<td>10.2</td>
<td></td>
<td>94.8</td>
<td>4.6</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>SD</td>
<td>1.7</td>
<td></td>
<td>3.1</td>
<td>3.3</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Figure 3-16  Control analysis of the effect of the $V_{\text{max}}$ for uptake or the $V_{\text{max}}$ for metabolism (0.1, 0.5, 2, 10, and 100 times value shown in table 3-2) on the steady state extraction ratio of EA ($E_{\text{ss}}$).
Use of cell-free perfusion circumvented the complexity of red blood cell glutathione conjugation of EA and was without effect on the movement of GSH (Ballatori et al., 1989), and the erythrocyte-free perfused liver was viable and stable (Fig. 3-12). In this preparation, hepatic removal of EA was concentration-dependent, and increasing the plasma concentrations of EA decreased its extraction ratio and resulted in saturation of EA-SG formation (Fig. 3-15). GSH conjugation of EA accounted almost completely for the total removal of EA because unchanged EA was found minimally in bile (Table 3-3). This observation is in disagreement with other reports that suggest extensive biliary excretion of unconjugated EA (Klaassen and Fitzgerald, 1974, Wallin et al., 1978, Peterlik et al., 1980), perhaps due to the lack of stabilization of the EA conjugates. Only a small proportion (~5%) of the formed EA-SG was further metabolized to EA-CG, EA-CYS and EA-NAC (Table 3-3), confirming that the liver is fully capable of mercapturate formation (Hinchman et al., 1991). Overall, little of the EA-SG effluxed into venous plasma, and the majority of EA-SG was excreted into bile (~89%) (Fig. 3-12C), resulting in the well known choleretic action of EA. Of note is that each micromole of excreted EA-SG elicited ~20 μl of bile flow, a value comparable to the apparent choleretic activity of bile acids (Roda et al., 1993).

The extraction ratio of EA was high ($E_{ss} \sim 0.8$ at the lowest concentrations examined) despite the high degree of protein binding to albumin ($f_u = 0.02 - 0.07$). As noted by us and others (Proost et al., 1993), liver models which include instantaneous equilibration in plasma protein binding and assume transmembrane movement of the free drug are incapable of predicting kinetic observations on EA. The enhancement of cellular uptake by an 'albumin receptor' mechanism was used as an explanation, but the fast dissociation on BSA binding (Weisiger, 1985; Nijssen et al., 1994) affords a better explanation for the observation. But when we employed a model similar to that described by Proost et al. (1993) to describe disequilibrium of EA binding within the sinusoids, we were unable to achieve adequate fits of the perfusion data.

To fully interpret the perfusion data, we employed a physiologic model of EA disposition that encompasses carrier-mediated uptake, bimolecular enzyme kinetics, co-substrate homeostasis, and efflux of the formed metabolite into bile and plasma. This model was relatively successful in
describing the dynamic removal of EA and the efflux of the GSH conjugate during rat liver perfusions at different drug input concentrations, and suggests that the excretion of EA-SG was an efficient process (Figs. 3-13C and 3-15B). Biliary excretion of EA-SG is a result of primary active transport by the canalicular multispecific organic anion transporter (cMOAT/MRP2) (Evers et al., 1998), and the minor efflux of EA-SG into plasma is attributed to the active transport by the multidrug resistance protein (MRP1) (Zaman et al., 1997) that is normally found at low levels on the hepatocyte basolateral membrane (Roelofsen et al., 1997). Efficient canalicular efflux of EA metabolites is necessary to minimize the well known product inhibition of GSTs seen for many glutathione S-conjugates, including EA-SG (Ploemen et al., 1990, Takamatsu and Inaba, 1992, Awasthi et al., 1993). The model predicted the time-course of perfusion at the higher EA concentrations (50 to 200 μM), during which loss of constancy in drug removal and diminished GSH conjugation were observed (Fig. 3-13). In these instances, rapid depletion of cellular GSH occurred (Figs. 3-13C and 3-14) because the capacity for GSH replenishment was lower than its consumption rate. Thus when hepatic GSH content fell to levels approaching the whole organ $K_m^{GSH}$ for GSH conjugation (~0.2-0.3 μmol/g liver according to Snel et al., 1995), 0.5 μmol/g liver according to Polhuijs et al. (1992), or 1.2 mM in this study, the rate limiting factor for GSH conjugation of EA changed from uptake to co-substrate availability.

There may be reasons for the departure of the model. It is probable that cellular EA-SG accumulation occurs especially at high input concentrations, and there remains the possibility that product inhibition could affect the removal of EA in liver. The model views the liver as a single compartment and fails to account for either zonal heterogeneity in EA uptake or GSH conjugation. factors that can affect local EA concentrations in the sinusoid and in whole liver (Kwon and Morris, 1997). Given that GSTs are localized predominantly in the perivenous region of sinusoids (Sippel et al., 1996) and that heterogeneity of transporters for uptake could exist, fits to the venous appearance of EA and EA-SG may be improved by including additional zonal and non-linear elements which describe EA-SG cellular efflux. Additionally, EA-SG formation may be described by multiple enzyme rate equations because different hepatic GSTs are involved (Ploemen et al.,
1993). Inclusion of the above refinements is prohibitive because this would lead to over-parameterization that ultimately leads to parameter unidentifiability for the limited set of data.

Fitting of the EA-SG formation rate vs. the logarithmic average EA concentration yielded a $K_m^{\text{overall}}$ of 67 $\mu$M and $V_{\text{max}}^{\text{overall}}$ of 0.23 $\mu$mol/min/g liver (Fig. 3-15B). These constants characterize the GSH conjugate formation at the whole organ level and do not reveal the events (uptake or conjugation) important to removal nor the rate-limiting process. Given the rapid spontaneous conjugation at physiologic levels of GSH and the remarkable rate-enhancement by the GSTs in rat liver cytosol (in vitro $V_{\text{max}} = 320$ $\mu$mol/min/liver assuming 60 mg cytosolic protein/g liver; Fig. 3-8B), EA-SG formation appears extremely rapid, and the overall GSH conjugation of EA is rate-determined mainly by uptake. The fitted results on the perfusion data with the physiologic model, however, predicts rate-modulation by metabolism (fitted $V_{\text{max}}^{\text{metab}} = 3.2$ $\mu$mol/min/liver). At the lowest (10 $\mu$M) and highest (200 $\mu$M) EA concentrations used for perfusion, the corresponding influx clearances at the basolateral membrane are ~100 and 40 ml/min (Eq. 3-3). If all EA entering the liver cell were available for metabolism, the $E_u$'s are expected to be ~0.80 and 0.62 at the low and high concentrations, respectively. The experimentally determined values were 0.8 and 0.45 (see Table 3-3), suggesting that at low EA concentrations, uptake is rate-limiting, whereas at high EA concentrations, metabolism is emerging to play a rate-limiting role. Hence, a control analysis was performed by varying the $V_{\text{max}}$'s for uptake and metabolism (0.01 to 100 times their mean values) at EA concentrations of 10, 50, and 200 $\mu$M (Fig. 3-15). The slopes of the change in $E_u$ at the 10 and 50 $\mu$M were steeper than that for 200 $\mu$M upon alteration of the $V_{\text{max}}$ for uptake than with the $V_{\text{max}}$ for metabolism. The analysis clearly infers that uptake is rate-limiting in the overall hepatic removal of EA at low concentrations, while at higher concentrations, uptake and metabolism both modulate the overall elimination of EA. The conclusion is further substantiated by the similarity in the constants for overall GSH conjugation and hepatocytic uptake.

In conclusion, we have shown that rapid GSH conjugation of EA occurs enzymatically and nonenzymatically. Rapid hepatic uptake of EA occurs despite high plasma protein binding and is facilitated by a sodium-independent carrier. Comparisons between the in vitro and whole organ
data, together with analysis based on a physiologic kinetic model, suggests that hepatic removal of EA by the erythrocyte-free perfused liver is rate-limited by cellular uptake. When hepatocyte GSH becomes depleted, as with perfusion with high concentrations of EA, co-substrate availability becomes the rate-limiting step in elimination.

3.7 APPENDIX

The following mass balance rate equations are used in the physiological model (Fig. 3-6). The description of parameters is given in Table 3-2.

The equations describing the liver plasma space for EA and EA-SG are

\[
\frac{d [EA_p]}{dt} = \left\{ C_n Q - [EA_p] Q - [EA_p] P_{diff} - \frac{V_{\text{uptake}} \cdot [EA_p]}{V_p} \right\} / V_p
\]  \hspace{1cm} (3-A1)

\[
\frac{d [EA-SG_p]}{dt} = \left\{ [EA-SG_c] P_{diff} [EA-SG] - [EA-SG_p] Q \right\} / V_p
\]  \hspace{1cm} (3-A2)

whereas the equations describing the cellular space are

\[
\frac{d [EA_c]}{dt} =
\]

\[
\frac{d [EA-SG_c]}{dt} =
\]

\[
\frac{d [EA-SG_c]}{dt} = \frac{V_{\text{metabol}} \cdot [EA_c] [GSH_c]}{K_m^GSH K_m^EA + K_m^GSH [EA_c] + [EA_c] [GSH_c]} - \left\{ [EA-SG_c] (P_{diff} [ES-SG] + CL_b [ES-SG]) \right\} / V_c + k_2 [EA_c] [GSH_c]
\]  \hspace{1cm} (3-A4)
\[
\frac{d[GSH]}{dt} = \left\{ K_m - \frac{V_{\text{max}} [EA][GSH]}{K_m^{EA} + K_m^{GSH} [EA] + K_m^{GSH} [GSH]} \right\} \left( \frac{1}{V_p - k_c [EA][GSH]} \right)
\]

where \([EA_p], [EA_c], [EA-SG_p], [EA-SG_c]\) are the concentrations of EA and EA-SG in the expanded plasma (sinusoidal plasma + Disse space) space and venous plasma (subscript "p") and cell (subscript "c"), respectively; \(V_p\) and \(V_c\) are the extracellular and cellular spaces, respectively; \([GSH]\) is the cellular GSH concentration; \(k_c\) is the constant for the non-enzymatic, bimolecular reaction; \(P_{\text{aff}}\) and \(P_{\text{aff}}(EA-SG)\) are the nonsaturable transport clearances at the sinusoidal membrane, respectively, for EA and EA-SG; \(CL_v(EA-SG)\) is the biliary intrinsic clearance of EA-SG. Other kinetic parameters for uptake and GSH homeostasis have been defined in the text.

### 3.7 STATEMENT OF SIGNIFICANCE OF CHAPTER 3

In this chapter we defined the \textit{in vitro} parameters describing the spontaneous and rat liver cytosol enhanced GSH conjugation of EA. Furthermore, kinetics of EA uptake by isolated rat hepatocytes revealed the presence of a saturable transport mechanism. With rat liver perfusion experiments, analysis of the data with a physiological kinetic model suggest that the rate limiting step in the hepatic GSH conjugation of EA is cellular uptake, but co-substrate availability rate limits metabolism when GSH is depleted. We also demonstrated the utility of \textit{in vitro} uptake and metabolism data for the description of elimination at the whole organ level.

### 3.8 ACKNOWLEDGMENTS

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CHAPTER 4

UPTAKE AND GLUTATHIONE CONJUGATION OF ETHACRYNIC ACID AND EFFLUX OF THE GLUTATHIONE ADDUCT BY PERIPORTAL AND PERIVENOUS RAT HEPATOCYTES

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4.1 ABSTRACT

The impact of zonal factors on the hepatic GSH conjugation of ethacrynic acid (EA) was assessed. Uptake of EA by enriched periportal (PP) and perivenous (PV) rat hepatocytes was characterized by both saturable \( V_{\text{max}}^{\text{uptake}} \) of 3.4 ± 1.7 and 3.2 ± 0.8 nmol/min/mg protein and \( K_{\text{m}}^{\text{uptake}} \) of 51 ± 13 and 44 ± 15 μM) and nonsaturable (12 ± 5 and 12 ± 3 μl/min/mg protein) components. Values for the overall GSH conjugation rates of EA (200 μM) were similar among the zonal hepatocytes, and resembled those for the influx transport rates. In absence of the hepatocyte membrane, GSH conjugation in PV and PP hepatocyte cytosol was similar, but a higher perivenous GSH conjugation activity towards EA (PV:PP of 2.4) that mirrored the higher PV:PP ratios of immunodetectable GSTs Ya (1.7) and Yb2 (2.5) was found in cell lysates obtained by the dual-digitonin-pulse perfusion technique. The GSH conjugation rates in the subcellular fragments were, however, much greater than those observed for intact hepatocytes. Efflux rates of the glutathione conjugate, EA-SG, from zonal hepatocytes were similar, as were levels of the immunodetectable multidrug-resistance protein 2/canalicular multispecific organic anion transporter (Mrp2/cMoat) in the 100,000g pellets. The composite results suggest that the GSTs responsible for EA metabolism are more abundant in the PV region, albeit that the gradient of enzymatic activities is shallow. Despite the existence of zonal metabolic activity, the overall GSH conjugation rate of EA is homogeneous among cells because the reaction is rate-limited by uptake, which occurs evenly. Results on EA-SG efflux suggest the acinar homogeneity in Mrp2/cMoat function for canalicular transport.

4.2 INTRODUCTION

Glutathione (GSH) conjugation is an important detoxification pathway for electrophiles, including some therapeutic agents, carcinogens, and reactive metabolites. Several factors impact on the rates of GSH conjugation in liver. These include the cellular uptake or in situ formation of acceptor substrates of GSH, the propensity for spontaneous GSH conjugation, the presence of the
glutathione S-transferases (GSTs), and co-substrate (GSH) availability. Once formed, the GSH-adduct is effluxed out of the hepatocyte by members of the multidrug resistance protein (MRP) family found either at the canalicular or basolateral membrane (Büchler et al., 1996; Keppler et al., 1998; Hirohashi et al., 1998; König et al., 1999). These processes are exemplified by the GSH conjugation of ethacrynic acid (EA) in vitro and in the perfused rat liver (Tirona and Pang, 1999). Data derived from in vitro spontaneous and enzymatic GSH conjugation and hepatocytic uptake of EA had been successfully scaled-up to describe observations from perfused rat liver with use of a physiologic, kinetic model that encompassed transport and bimolecular, spontaneous (non-enzymatic) and enzymatic metabolism. The data suggest that hepatic uptake rate-limited the GSH conjugation of EA at low input concentrations (< 50 μM), whereas cellular metabolism played an increasingly greater role at higher concentrations (~200 μM). As intracellular levels of GSH became depleted as a result of consumption, co-substrate availability invariably rate-limited GSH conjugation.

There is, additionally, an increasing awareness that acinar heterogeneity in sinusoidal uptake and metabolism affects hepatic drug processing (Pang, 1995). Kwon and Morris (1997) demonstrated, in theory, that the total hepatic elimination of drugs would be influenced by zonal localization of transport and enzymatic activities. For GSH conjugation that is a bimolecular reaction, there is the extra consideration of the unevenness in cellular availability of the cosubstrate that could further affect the overall hepatic removal (Pang, 1995). There exists much evidence that the GSTs are more concentrated in the perivenous (PV) than the periportal (PP) regions (Redick et al., 1982). The rat liver GSTs that are constitutively expressed and capable of metabolizing EA (Ploemen et al., 1991, 1993) such as the Alpha (subunits 1, 2 and 8) and Mu (subunits 3 and 4) classes are also more abundant in the PV zone (Sippel et al., 1996). Other GSTs - the microsomal GSTs and those belonging to the Theta class - exhibit negligible activity towards EA (Ploemen et al., 1993), and these are also constitutive to the PV region (Mainwaring et al., 1996). The transport of EA into isolated rat hepatocytes was facilitated by a sodium-independent transporter whose identity and zonal distribution is currently unknown, and by a nonsaturable (linear) system (Tirona...
and Pang, 1999). It is conceivable that zonal uptake exists for EA because this zonated event occurs within PV hepatocytes for substrates such as cysteine (Saiki et al., 1992), glutamate (Burger et al., 1989; Tan et al., 1999), and α-ketoglutarate (Moseley et al., 1992). Likewise, predominance of immunodetectable sinusoidal transport proteins was observed in PV hepatocytes for the rat glucose transporter 1 (GLUT1) (Tal et al., 1990), the rat organic cation transporter 1 (rOCT1) (Meyer-Wentrup et al., 1998) and the rat organic anion transporting polypeptide 2 (oatp2) (Kakyo et al., 1999). By contrast, acinar homogeneity exists for the rat sodium-dependent taurocholate transporting polypeptide, ntcp (Stieger et al., 1994; Tan et al., 1999) and the organic anion transporting polypeptide from rat liver, oatp1 (Abu-Zahra et al., 1999) in their uptake of substrates.

In this communication, the initial rates of uptake of EA were investigated and the possible roles of acinar metabolism and transport by isolated, enriched PP and PV rat hepatocytes were explored. We further studied accumulation of the formed GSH adduct of EA (EA-SG) because it is recognized that GSH conjugates, including EA-SG, are product-inhibitors of GSTs (Ploemen et al., 1990), and the efficiency in efflux of GSH conjugates may play an important role in the cellular detoxication of electrophiles by the GSTs. The transporters responsible for this basolateral efflux of EA-SG in the rat have not been directly identified but are likely orthologs of human MRP family proteins (Zaman et al., 1997). However, considerably faster efflux of EA-SG occurs with the canicular multispecific organic anion transporter, cMoat or Mrp2 (Büchler et al., 1996; Evers et al., 1998), and this accounted for the rapid appearance of EA-SG in rat bile (Tirona and Pang, 1999). However, it is unknown whether acinar heterogeneity exists for the sinusoidal or canicular efflux processes in rat liver.

4.3 MATERIALS AND METHODS

4.3.1 Materials. [14C]EA (specific activity 15 mCi/mmol) was a kind gift from Dr. J.H.T.M. Ploemen (TNO, Ziest, The Netherlands) and was purified by HPLC and solid phase extraction (radiochemical purity >98%). [3H]Sucrose (specific activity 10 Ci/mmol) was obtained from NEN Life Sciences (Boston, MA). EA, 1-chloro-2,4-dinitrobenzene (CDNB), GSH and oxidized GSH
(GSSG) were purchased from Sigma (St. Louis, MO). EA-SG was synthesized as described previously (Tirona and Pang, 1999). Digitonin was obtained from Fluka Chemie (Buchs, Switzerland). Collagenase was purchased from Boehringer Mannheim (Oakville, ON). Antisera raised against rat GST Ya (rGST 1-1) and Yb2 (rGST 4-4) were obtained from Biotrin International (Dublin, Ireland). Polyclonal antibodies against rat MrpUcMoat (EAG 15, Büchler et al., 1996) and monoclonal antibodies against rat cytochrome P450 1A (CYP1A) isozymes (MAb 1-7-1) were generously provided by Drs. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany) and H.V. Gelboin (National Institutes of Health, Bethesda, MD), respectively. All other reagents were the highest available grade.

4.3.2 Isolation of Periportal (PP) and Perivenous (PV) Rat Hepatocytes. Enriched PP and PV hepatocytes from male, Sprague-Dawley rats (275-325 g, Charles River Canada, St. Constant, PQ) were harvested by the digitonin/collagenase perfusion method according to Lindros and Penttilä (1985), with slight modifications as detailed by Tan et al. (1999). Briefly, rat livers were perfused via the portal vein at a flow rate of 25 ml/min with oxygenated, Ca²⁺-free, Hanks medium (pH 7.2) supplemented with 10 mM HEPES, 0.5 mM EGTA, 4.2 mM NaHCO₃, 5 mM glucose and 0.65% BSA. Next, the perfusate was changed to the digitonin solution containing 3.25 mM digitonin, 150 mM NaCl, 6.7 mM KCl and 50 mM HEPES and delivered at a flow rate of ~6 ml/min via the portal or hepatic vein until the desired zonal destruction pattern was observed on the surface of the liver. The direction of flow was reversed and the liver perfused with Ca²⁺-free buffer for 2 min followed by collagenase buffer (Hanks buffer supplemented with 4 mM CaCl₂ and 0.06% collagenase) until the tissue was digested. Hepatocyte viability (>90%) was assessed by Trypan blue exclusion. Zonal enrichment was routinely estimated by monitoring the activities of alanine aminotransferase (ALT) with a commercially available kit (Sigma) and glutamine synthetase (GS) by a standard UV method (Tan et al., 1999). Protein was determined by the method of Lowry et al. (1951). Several low speed centrifugations (50g) during the isolation procedure separated most of the non-parenchymal cells from hepatocytes; it was surmised that low-levels of contamination persisted.
4.3.3 Uptake of EA by PP and PV Rat Hepatocytes. The zonal hepatocytes were suspended in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with glucose (5 mM) and HEPES (1 mM), and were preconditioned for 10 min at 37°C. A mixture of [¹⁴C]EA, [³H]sucrose, and unlabeled EA was added to the cell suspension to result in final EA concentrations of 1 to 800 μM and ~1.67 x 10⁶ cells/ml. Samples were retrieved at 15 to 20 sec intervals after admixture and were rapidly centrifuged through a layer of silicon oil as described (Tan et al., 1999). Cellular radioactivity was determined by liquid scintillation spectrometry (LSC, Model 5801, Beckman Canada, Mississauga, ON), after correction of the adhered water layer defined by [³H]sucrose (Tan et al., 1999). Because the initial cellular accumulation of EA was linear over 80 sec, the rate of uptake, \( v_{uptake} \), was estimated as the slope upon regression of the accumulated amount vs. time data. The kinetics of EA uptake were analyzed by fitting \( v_{uptake} \) against the initial substrate concentration [EA] with use of equation (Eq. 4-1, after eliminating other possibilities of single and multiple saturable systems) and appropriate weighting scheme with a least-squares fitting routine by the software, SCIENTIST (Micromath Scientific Software, Salt Lake City, UT).

\[
v_{uptake} = \frac{V_{max} [EA]}{K_m + [EA]} + P_{diff} [EA]
\]

where \( V_{max} \) and \( K_m \) are the maximum uptake rate and the Michaelis-Menten constant for the saturable process and \( P_{diff} \) is the uptake clearance for the linear component.

4.3.4 Metabolism of EA by PP and PV Rat Hepatocytes. We chose to study EA at the concentration of 200 μM because GSH conjugation of the drug would be influenced by both cellular uptake and enzymatic activity (Tirona and Pang, 1999). Hence, inherent acinar differences of these factors would be more apparent. EA, dissolved in physiological saline solution, was added to the hepatocyte suspension to achieve an initial concentration of 200 μM with ~1.67 x 10⁶ cells/ml. At 1 to 5 min intervals throughout the 20 min incubation experiment conducted at 37°C, samples (500 μl) were retrieved and placed into 1.5 ml microcentrifuge tubes containing 70 μl of 70% perchloric acid. A separate sample (700 μl) was overlaid atop 250 μl of 1-bromododecane and centrifuged (Biofuge pico, Heraeus Instruments, Germany) for 10 sec. An aliquot (500 μl) of the
resulting supernatant was similarly placed into 70% perchloric acid to assay for contents in the extracellular space of the incubation mixture. The acidified samples were immediately mixed and stored at -70°C until analysis.

**4.3.5 Analysis.** For the quantitation of EA and EA-SG, each acidified sample (total incubation mixture and extracellular medium) was further combined with 200 μl of 1.2 mM 4-(2,4-dichlorophenoxy)-butyric acid (internal standard). After centrifugation, 100 μl of the supernatant was analyzed by HPLC according to a previously developed procedure (Tirona and Pang, 1999). Standard curves prepared from solutions of known concentrations of EA (50 - 250 μM) and EA-SG (10 - 250 μM), were constructed in a similar fashion.

For the analysis of GSH and GSSG, 500 μl of the total suspension (cell and extracellular medium) and 500 μl of the extracellular medium were added to microcentrifuge tubes containing 100 μl of 70% perchloric acid and 50 μl of 15 mM bathophenanthroline-disulfonic acid. After centrifugation, the supernatant was immediately analyzed for GSH and GSSG with use of the derivatization and HPLC method of Fariss and Reed (1987). Acid-precipitated hepatocytes were further analyzed for the GSH-protein mixed disulfide content with the same HPLC method (Fariss and Reed, 1987). The intracellular concentrations of EA, EA-SG and GSH/GSSG were estimated as the difference between the concentrations in the total suspension and the extracellular medium.

**4.3.6 GST Activity of PP and PV Hepatocyte Cytosols.** Cytosol was obtained by homogenization of the zonal hepatocytes (Ultra-Turrax T25 homogenizer, Janke & Kunkel, Staufen im Briesgau, Germany) and the resultant supernatant fractions were sequentially centrifuged at 9000g and 100,000g at 4°C. Cytosolic GST activity towards EA was determined within the linear protein concentration range by the spectrophotometric method of Satoh (1995) as previously described (Tirona and Pang, 1999) with 200 μM of EA and 5 mM of GSH, at 37°C and pH 7.2. Cytosolic GST activity towards CDNB was determined by standard UV methods with 1 mM CDNB and 1 mM GSH, at 25°C and pH 6.5 (Habig et al., 1974). The GST-catalyzed GSH conjugation activities towards EA and CDNB were obtained after correction of the (total) cytosolic
GSH conjugation rates for the spontaneous reaction rates (in absence of cytosol) and normalization to the protein contents.

4.3.7 PP and PV Cell Lysate. Preparation of the cell lysates from the most proximal and distal hepatocytes along the sinusoidal plate was performed according to the dual-digitonin-pulse perfusion method of Quistorff and Grunnet (1987), with modifications. Paired zonal (PP and PV) lysates are prepared from the same liver. Rat livers were perfused with Hank’s buffer (pH 7.2) containing 10 mM HEPES, 0.5 mM EGTA, 4.2 mM NaHCO₃, and 5 mM glucose (perfusion buffer) pregassed with 95% O₂/5% CO₂ at a flow rate of 30 ml/min into the portal vein in a prograde fashion. After 10 min, the flow rate was reduced to 12 ml/min, and the direction of flow was reversed (retrograde perfusion into the hepatic vein). After stabilization of the liver for 1 min, the perfusion medium was changed to the digitonin solution (3.25 mM digitonin, 150 mM NaCl, 6.7 mM KCl and 50 mM HEPES) for perfusion at 6 ml/min until a spotted destruction pattern was observed on the liver surface (35 ± 11 sec) for destruction of the PV region. The flow was then reverted to prograde flow, and perfusion buffer was used for perfusion at a rate of 20 ml/min. The eluate (PV lysate) was collected over 30 sec. For continued preparation of the PP lysate, the direction of flow was reversed. Rat livers were perfused with perfusion buffer at the flow rate of 20 ml/min into the hepatic vein in a retrograde fashion for 2.5 min. Then the flow rate was reduced to 12 ml/min for 1 min. Next, the digitonin solution was infused at 6 ml/min until a circular destruction pattern appeared (26 ± 8 sec) for destruction of the PP region. Subsequently, the eluate (PP lysate) was collected for 30 sec. PP and PV eluates were centrifuged at 100,000g for 60 min at 4°C and the resultant supernatants were stored at -70°C.

4.3.8 Kinetic Modeling of EA Disposition by PP and PV Hepatocytes. A kinetic model, whose scheme is shown in Fig. 4-1, was employed for analysis of the time-dependent disposition of EA in PP and PV hepatocytes. The saturable uptake (Kₘmax and Vₘmax) and the bi-directional (uptake and efflux) linear clearance (Pₐₒ) parameters, obtained from uptake experiments (Fig. 4-2), were used to denote transmembrane transport. The spontaneous and GST-catalyzed GSH conjugations of EA were described by a second-order (spontaneous) reaction and a single enzyme-
catalyzed, rapid-equilibrium, random sequential order scheme as reported previously (Tirona and Pang, 1999). The second-order constant \( k_2 \) for the spontaneous reaction and the apparent Michaelis-Menten constants for GSH \( \left( K_m^{GSH} \right) \) and EA \( \left( K_m^{EA} \right) \) for the enzymatic reactions were obtained from the previous \textit{in vitro} studies (Tirona and Pang, 1999). The maximal enzymatic conjugation rate \( V_{\text{max}} \) was estimated by the fitting procedure.

EA-SG formed within isolated hepatocytes was assumed to undergo net efflux into the extracellular medium and transport into intracellular sequestration vesicles (see discussion). These linear clearance processes, denoted by the terms, \( CL_{\text{efflux}}^{EA-SG} \) and \( CL_{\text{ex}}^{EA-SG} \), respectively, were estimated by fitting. Because control experiments indicated a lack of net change (over 20 min) in intracellular hepatocyte GSH (Figs. 4-3A and 4-4A) and because GSH synthesis is expected to be insignificant in absence of GSH precursors under the experimental conditions, intracellular GSH kinetics were simplified - the loss of GSH was due only to conjugation. However, there was lack of mass balance because the amount of GSH consumed was less than the total amount of EA-SG formed. The intracellular GSH concentrations were scaled-up to allow for mass balance for the purposes of fitting; the GSH data were converted to concentration terms by assuming 10 \( \mu \)l of cell volume/ml of suspension. The mean data of the PP and PV hepatocyte experiments were simultaneously fitted with the model equations with use of a nonlinear least squares procedure (SCIENTIST) and appropriate weighting schemes. The fitted parameter values, together with the assigned parameters, are shown in Table 4-3.

The following mass balance rate equations are used in the model (Fig. 4-1).

The equations describing the extracellular space (EC) for EA and EA-SG are

\[
\frac{d[EA]_{EC}}{dt} = \left( -\frac{V_{\text{spade}}^{\text{max}} [EA]_{EC}}{K_m^{EA}} + P_{\text{d}}[EA]_{EC} + P_{\text{d}}[EA]_{C} \right) / V_{EC} \tag{4-2}
\]

\[
\frac{d[EA-SG]_{EC}}{dt} = (CL_{\text{efflux}}^{EA-SG} [EA-SG]_{C}) / V_{EC} \tag{4-3}
\]

whereas the equations describing the cellular space (C) are
\[
\frac{d[EA]_c}{dt} = \left(\frac{V_{\text{max}}[EA]_{EC}}{K_m^{\text{metabolite}} + [EA]_{EC}} + P_{\text{deg}}[EA]_{EC} - P_{\text{deg}}[EA]_c - k_2[EA]_c[GSH]_c V_c\right) - \frac{V_{\text{metabolite}}[EA]_c[GSH]_c}{K_m^{GSH} K_m^{EA} + K_m^{GSH} [GSH]_c + K_m^{EA} [EA]_c + [EA]_c[GSH]_c} / V_c \tag{4-4}
\]

\[
\frac{d[EA-SG]_c}{dt} = (k_2[EA]_c[GSH]_c V_c - \frac{V_{\text{metabolite}}[EA]_c[GSH]_c}{K_m^{GSH} K_m^{EA} + K_m^{GSH} [GSH]_c + K_m^{EA} [EA]_c + [EA]_c[GSH]_c}) / V_c - CL_{\text{efflux}} [EA-SG]_c - CL_{\text{ves}} [EA-SG]_c / V_c \tag{4-5}
\]

\[
\frac{d[GSH]_c}{dt} = (-k_2[EA]_c[GSH]_c V_c - \frac{V_{\text{metabolite}}[EA]_c[GSH]_c}{K_m^{GSH} K_m^{EA} + K_m^{GSH} [GSH]_c + K_m^{EA} [EA]_c + [EA]_c[GSH]_c}) / V_c \tag{4-6}
\]

and the equation which describes the accumulation of EA-SG within the vesicular sequestration space (V) is

\[
\frac{dEA-SG_v}{dt} = CL_{\text{ves}} [EA-SG]_c \tag{4-7}
\]

where \([EA], [EA-SG],\) and \([GSH]\) are the concentrations of EA, EA-SG and GSH in each of the compartments (subscripts EC, C, and V for extracellular, cellular and vesicular spaces, respectively). Total intracellular EA-SG is represented by the sum of cellular and vesicular contents.

4.3.9 Immunoblot Analysis. Cytosols, lysates (5 μg protein containing the GSTs) and 100,000g pellets (10 μg protein containing Mrp2 and the PV marker protein, CYPIA2) derived from PP and PV hepatocytes were used for analysis. The immunoreactive proteins were resolved by sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE) on 12, 9 and 7.5% gels for the GSTs, CYPIA2 and Mrp2, respectively, using the MiniProtean II system (BioRad; Mississauga,
Protein was transferred onto nitrocellulose membranes (Hybond ECL: Amersham, Oakville, ON) with a semi-dry transfer unit (BioRad). Subsequently, the membranes were blocked with 10% non-fat milk in Tris-buffered-saline with 0.1% Tween 20 (TBST) for 2 h at room temperature. After washing with TBST, membranes were incubated with primary antibody (anti-GST Ya or Yb2 at 1:50,000 dilution, MAb 1-7-1 at 1:20,000 dilution, and EAG15 at 1:40,000 dilution) in TBST overnight at 4°C. Following washing with TBST, the membranes were incubated with horseradish peroxidase-linked anti-rabbit (GST and Mrp2) or anti-mouse immunoglobulins (CYP1A2)(Amersham or BioRad) at 1:20,000 dilution in TBST for 2 h at room temperature. Detection was performed using enhanced chemiluminescence (ECL, Amersham), and membranes were visualized on Hyperfilm (Amersham). For the semi-quantitation of the GSTs, CYP1A2, and Mrp2, the films were scanned (Umax Astra 1200S) and band intensities were integrated using the NIH Image software (developed at the National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Figure 4-1  Model for GSH conjugation of EA in isolated hepatocytes. Cellular EA uptake is represented by saturable and linear (bi-directional) processes. GSH conjugation is modeled using second-order spontaneous and bimolecular enzymatic reactions. Net EA-SG efflux into the extracellular medium and into a sequestered vesicular space are described by linear clearances. See text for details.
4.3.10 **Statistical Analysis.** All data are presented as mean ± standard deviation. Unpaired and paired Student's t-tests were used where appropriate; the level of significance was set at 0.05.

4.4 **RESULTS**

4.4.1 **Biochemical Characterization of PP and PV Hepatocytes and Lysates.** The activities of PP and PV marker enzymes (ALT and GS, respectively) are summarized in Table 4-1. Significant differences were observed in marker enzyme activities indicating the attainment of the zonal enrichment of PP and PV hepatocytes \((p < .05)\) and PP and PV lysates \((p < .05)\). The PP/PV activity ratios for ALT were higher for lysate (7.5) than for hepatocyte cytosol (1.8), confirming the steeper and decreasing (portal to venous) acinar gradient in enzyme content. The PP/PV activity ratios for GS were similar among lysates and hepatocyte cytosols (0.025 and 0.029 respectively) and these results are consistent with the confined localization of this enzyme to the terminal 2 to 3 hepatocytes in the PV region. These observations established the validity of the preparation on the enrichment of PP and PV hepatocytes or lysates.

<table>
<thead>
<tr>
<th>Table 4-1. <strong>Marker enzyme activities of PP and PV hepatocytes and lysates.</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hepatocytes</strong>^a^</td>
</tr>
<tr>
<td>( \text{ALT Activity (nmol/min/mg protein)} )</td>
</tr>
<tr>
<td>PP (n=13)</td>
</tr>
<tr>
<td>PV (n=13)</td>
</tr>
<tr>
<td>PP/PV</td>
</tr>
<tr>
<td>ANOVA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Lysates</strong>^b^</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ALT Activity (nmol/min/mg protein)</strong></td>
</tr>
<tr>
<td>PP(n=4)</td>
</tr>
<tr>
<td>PV(n=4)</td>
</tr>
<tr>
<td>PP/PV</td>
</tr>
<tr>
<td>ANOVA</td>
</tr>
</tbody>
</table>

^a^ activities obtained from 13,000g supernatant

^b^ activities obtained from 100,000g supernatant
4.4.2 Concentration-Dependent Uptake of EA by Zonal Hepatocytes. EA uptake kinetics were similar among PP and PV hepatocytes (Fig. 4-2). The uptake parameter estimated upon fitting of the data to Eq. 4-1 furnished similar $K_{m}^{uptake}$ (51 ± 13 and 44 ± 15 µM) and $V_{max}^{uptake}$ (3.4 ± 1.7 and 3.2 ± 0.8 nmol/min/mg protein) values for the saturable uptake of EA for PP and PV hepatocytes ($p > .05$, n=4); comparable values were also obtained for the nonsaturable component. $P_{&dagger;}$ (12 ± 5 and 12 ± 3 µl/min/mg protein) for PP and PV hepatocytes ($p > .05$, n=4).

4.4.3 Cellular GSH Concentration and GSH Conjugation Rates of EA by PP and PV Hepatocytes. Control (saline-treated) PP and PV hepatocytes (n=4) retained their initial intracellular GSH contents for at least 20 min, and the extracellular GSH levels remained constant throughout the incubation period (Figs. 4-3A and 4-4A, see insets). GSSG, found mainly in the extracellular space, remained virtually constant during the incubation and accounted for approximately 10 to 15% of the total GSH equivalents in the system.

The addition of EA (200 µM) to PP and PV hepatocyte suspensions (n=12) greatly reduced GSH levels that in turn affected the rate of GSH conjugation and disappearance of EA (Figs. 4-3A and 4-4A). Although the extracellular GSH levels remained constant throughout the incubation period in treated hepatocytes and were similar to those of the controls, the GSH within PP and PV hepatocytes was rapidly depleted within the first 5 min after EA treatment (Figs. 4-3A and 4-4A). The pattern of depletion rate was, however, independent of the acinar origin of the hepatocytes.

EA disappeared at similar rates in total suspension and the extracellular medium of the incubation system with PP and PV hepatocytes (Figs. 4-3B and 4-4B). Cellular accumulation of EA was low in either PP or PV hepatocytes. The loss of EA was completely accounted for by the formation of EA-SG, and no other sequential metabolite (cysteinyl-glycine, cysteine and N-acetyl-cysteine adducts) was detected. Essentially, mass balance was conserved by EA and EA-SG throughout the 20 min incubation period; the dose recovery was 100 ± 5% and 100 ± 6% for the PP and PV hepatocyte incubation systems, respectively.
Figure 4-2  Concentration-dependent uptake of \([^{14}\text{C}]\text{EA}\) by isolated rat PP (A) and PV (B) hepatocytes. The solid line (—) was obtained by fitting data with Eq. 4-1 where (— —) indicates the saturable component and (---) represents the non-saturable component for EA uptake (mean ± SD, n=4).
Figure 4-3  GSH conjugation of EA (200 μM) by isolated PP rat hepatocytes. GSH (A) EA (B), and EA-SG (C) contents as determined by HPLC (mean ± SD, n=6) in the total suspension, and extracellular and cellular spaces. The lines (——), (———), (———) represent the fits to the kinetic model for extracellular, intracellular and total species except for (A) where fits to the intracellular GSH are shown. The inset in (A) shows the GSH contents in control (saline-treated) PP hepatocytes (n=4).
Figure 4-4  GSH conjugation of EA (200 μM) by isolated PV rat hepatocytes. GSH (A), EA (B), and EA-SG (C) contents as determined by HPLC (mean ± SD, n=6) in the total suspension, and extracellular and cellular spaces. The lines (—), (— — —), (— — —) represent the fits to the kinetic model for extracellular, intracellular and total species except for (A) where fits to the intracellular GSH are shown. The inset in (A) shows the GSH contents in control (saline-treated) PV hepatocytes (n=4).
Both PP and PV hepatocytes produced EA-SG at equivalent rates (Figs. 4-3C and 4-4C). Because rapid efflux of EA-SG occurred upon its formation within cells, the formation rate of EA-SG was more appropriately estimated by viewing the early-in-time data for total concentration in the incubation system before significant depletion of cellular GSH occurred. The initial formation rates (roughly estimated from up slope of the data between 0 to 3 min) of 6.2 ± 0.9 and 6.9 ± 1.1 nmol/min/mg protein, respectively, were comparable (p > .05), and were similar to those observed for uptake (5.4 ± 1.5 and 5.2 ± 0.9 nmol/min/mg protein respectively; Table 4-2) at 200 μM. This suggests that GSH conjugation occurred very rapidly upon cellular uptake of EA. The EA-SG formation rate rapidly approached its asymptotic values by 10 min (Figs. 4-3C and 4-4C) due to the depletion of GSH (Figs. 4-3A and 4-4A). The EA-SG formed within the zonally-enriched hepatocytes rapidly escaped into the extracellular medium in a monoexponential fashion. After 15 min, EA-SG remained sequestered within the hepatocyte with incubation time, and the net efflux of cellular EA-SG approached zero. Because the efflux rate equaled the rate of accumulation in extracellular medium, the initial EA-SG efflux rate was estimated from the early-in-time data for the extracellular medium (1.5 to 5 min, excluding zero time due to the short lag-time involved with the cellular formation/cellular distribution/appearance of EA-SG in medium). These were similar among the zonal hepatocytes (2.2 ± 0.2 and 2.3 ± 0.3 nmol/min/mg protein, respectively, p >0.05) and the accumulation of EA-SG in extracellular medium approached their corresponding asymptotic values by 15 min. Although the rates of initial efflux of EA-SG were considerably slower than those of formation, the accumulation of intracellular EA-SG was short-lived because EA-SG formation ceased by 7.5 min due to the almost complete depletion of GSH (Figs. 4-3A and 4-4A).

It was noted that the total formation of the EA-SG (~25 nmol/mg protein) at the end of the experiment exceeded the total loss of cellular GSH (from ~15-17 nmol/mg protein to almost zero). Because the summed loss of EA equated with the production of EA-SG, it is unlikely that experimental errors occurred in the quantitation by HPLC. GSH synthesis within the hepatocytes could account for the difference because the rate of GSH synthesis by hepatocytes has been
estimated to be ~0.4 nmol/min/10⁶ cells (Lu et al., 1991). But this possibility is remote due to the lack of amino acid precursors in the suspension medium and the lack of net change in GSH in control hepatocytes (Figs. 4-3A and 4-4A, insets). Furthermore, total EA-SG formation ceased after 10 min, an observation that contradicts the sustained GSH synthesis. The levels of GSH-protein mixed disulfides, measured in a separate group of hepatocytes, accounted for only ~1% of the total GSH content. At this time, we have no explanation for this discrepancy.

Table 4-2. Summary of EA (200 μM) disposition by PP and PV rat hepatocytes.

<table>
<thead>
<tr>
<th></th>
<th>PP</th>
<th>PV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial EA uptake rate</td>
<td>5.4 ± 1.5</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td>Initial EA-SG formation rate</td>
<td>6.2 ± 1.0</td>
<td>6.9 ± 1.1</td>
</tr>
<tr>
<td>Initial EA-SG efflux rate</td>
<td>2.2 ± 0.2</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>In vitro EA-SG formation rate in cytosol</td>
<td>203 ± 16</td>
<td>211 ± 29</td>
</tr>
</tbody>
</table>

*Observed from [¹⁴C]EA hepatocyte uptake experiments.

*Total EA-SG formation rates was estimated from the slope of data points between 0 and 3 min subsequent to the addition of EA in the incubation mixture.

*EA-SG efflux rate into extracellular medium was estimated as the slope of data points between 1.5 and 5 min subsequent to the addition of EA in the incubation mixture.

* Determined by spectrophotometric assay for hepatocyte cytosolic fractions at 200 μM EA and 5 mM GSH, pH 7.2 and 37°C.

4.4.4 Fitting of Data to the Kinetic Model. Reasonable fits to the data were obtained upon scale-up of intracellular GSH to provide for mass-balance (Figs. 4-3 and 4-4). Values for the fitted $v_{max}$, $CL_{efflux}$ and $CL_{EASG}$ (Table 4-3) revealed that the processes governing the enzymatic formation of EA-SG and its transport were all similar among the zonal hepatocytes. We further tried out other kinetic models that do not include an intracellular sequestration space. However, all
failed to provide adequate fits to the observations because these predicted complete intracellular depletion of EA-SG by the end of the incubation experiment.

The fits showed that the initial rates of EA-SG formation were somewhat underestimated by the model (Figs. 4-3C and 4-4C) when parameters observed for EA transport were assigned with values obtained from the uptake experiments. These values, based on $[^{14}]$EA influx in rapid uptake studies (Fig. 4-2, Table 4-2), predicted a slightly lower initial rate of EA uptake that resulted in a more gradual decline in EA disappearance and lowered formation of EA-SG during the first 3 min of incubation (Figs. 4-3 and 4-4). A systematic trend occurred with the fitting of the cellular and extracellular EA-SG data (Figs. 4-3C and 4-4C), and might have been the consequence of lack of modeling of the time-dependent decrease in $CL_{er}^{EA-SG}$ and concomitant increase in $CL_{er}^{EA-SG}$ with progressive internalization of Mrp2 during the time-span of the experiment. Moreover, transport of EA-SG from cell to extracellular medium was described only by a net efflux clearance parameter ($C_{er}^{EA-SG}$) and failed to incorporate bi-directional movement.

The present data for efflux of EA-SG from hepatocytes were further scaled-up with the scaling factor ($\alpha/\beta$) (where $\alpha$ is $1.25 \times 10^8$ cells/g liver and $\beta$ is $1 \times 10^6$ cells/mg protein) and compared to that obtained from the whole organ (Tirona and Pang, 1999). The calculation was based on the assumption that the total efflux clearance for EA-SG in hepatocytes equaled the sum of $C_{er}^{EA-SG}$ and $CL_{er}^{EA-SG}$ ($\approx 1.2 \mu l/min/mg$), yielding an efflux clearance of 1.5 ml/min/10 g liver for whole liver. The estimated value was similar to the sum of sinusoidal and biliary clearance for EA-SG (1.1 ml/min/10 g liver) in perfused rat liver studies (Tirona and Pang, 1999).

The $V_{max}^{metab}$ estimates ($\approx 35$ nmol/min/mg) obtained with model fitting (Table 4-3) with the hepatocyte experiments were much lower than that observed for the GST activity in vitro (in cytosol) towards EA-SG formation from 200 $\mu$M EA and 5 mM GSH at physiological conditions ($\approx 200$ nmol/min/mg, Table 4-2). This big discrepancy was the result of the very poor reliability of $V_{max}^{metab}$ because only one concentration was studied and saturation of metabolism might not have
been attained under the experimental condition. These led to the large standard deviation of the estimate (Table 4-3).

4.4.5 GST Activities in Cytosolic Fractions of PP and PV Hepatocytes and Lysates. The \textit{in vitro} GST activities derived from cytosols of PP and PV hepatocytes and PP and PV lysates towards EA are summarized in Fig. 4-5A. No difference in GST activity was observed among zonally enriched hepatocytes, whereas a 2.4-fold greater activity ($p < 0.05$) was observed for the PV lysate compared to the PP lysate. The GST activities towards EA mirrored those towards CDNB (Fig. 4-5B). No difference in cytosolic GST activity was seen in zonal hepatocytes towards CDNB, whereas a 1.9-fold difference ($p < 0.05$) was observed between PV and PP lysates.

4.4.6 GSTs in PP and PV Hepatocytes and Lysates. The levels of two constitutive rat liver GSTs (Ya and Yb2) were found to be similar for the cytosolic fractions of PP and PV hepatocytes (Fig. 4-6) when these were assessed by SDS-PAGE and densitometry. However, the GST Ya and Yb2 proteins in PV lysate were 1.7- and 2.5-times those of the PP lysates (Fig. 4-7).

4.4.7 Mrp2 in PP and PV Hepatocytes. Levels of immunoreactive Mrp2 in crude membranes obtained from 100,000g pellets of homogenized PP and PV hepatocytes are shown in Fig. 4-8. PP and PV hepatocytes contained similar amounts of Mrp2 protein. By contrast, the same membrane fractions contained a highly variable and 3 to 4-fold enrichment of constitutively expressed CYP1A2 in PV hepatocytes ($p < 0.5$), as anticipated for the marker protein.
Table 4-3. Parameters for the kinetic model of EA disposition in isolated hepatocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{\text{uptake}}^m$</td>
<td>Michaelis-Menten constant for EA uptake</td>
<td>PP: 51, PV: 44</td>
</tr>
<tr>
<td>$V_{\text{uptake}}^{\text{max}}$</td>
<td>Maximum facilitated uptake velocity for EA</td>
<td>PP: 3.4, PV: 3.2</td>
</tr>
<tr>
<td>$P_{\text{diff}}$</td>
<td>Bi-directional transmembrane clearance for EA</td>
<td>PP: 12, PV: 12</td>
</tr>
<tr>
<td>$k_2$</td>
<td>Second-order spontaneous GSH conjugation constant</td>
<td>PP: 126</td>
</tr>
<tr>
<td>$K_m^{GSH}$</td>
<td>Michaelis-Menten constant for GSH in enzymatic reaction</td>
<td>PP: 94</td>
</tr>
<tr>
<td>$K_m^{EA}$</td>
<td>Michaelis-Menten constant for EA in enzymatic reaction</td>
<td>PP: 1.2</td>
</tr>
<tr>
<td>$V_{\text{metub}}^{\text{max}}$</td>
<td>Maximum enzymatic GSH conjugation rate</td>
<td>PP: 30 ± 20, PV: 40 ± 51</td>
</tr>
<tr>
<td>$CL_{\text{EA-SG efflux}}^{\text{compartment}}$</td>
<td>Net EA-SG efflux clearance into the extracellular compartment</td>
<td>PP: 0.98 ± 0.18, PV: 0.89 ± 0.19</td>
</tr>
<tr>
<td>$CL_{\text{EA-SG vesicle}}^{\text{sequestration compartment}}$</td>
<td>Net EA-SG efflux clearance into the vesicular sequestration compartment</td>
<td>PP: 0.28 ± 0.10, PV: 0.24 ± 0.11</td>
</tr>
<tr>
<td>$[GSH]_0$</td>
<td>Initial hepatocyte GSH concentration</td>
<td>PP: 6.9, PV: 8.0</td>
</tr>
</tbody>
</table>

* Obtained from [$^{14}$C]EA hepatocyte uptake experiments.
* From Tirona and Pang, 1999.
* Fitted value (± standard deviation of the estimate) using the kinetic model.
* Scaled-up from hepatocyte GSH measurements to maintain mass-balance.
Figure 4-5  GST activity towards EA (A) and CDNB (B) in cytosols prepared from isolated PP and PV rat hepatocytes (mean ± SD; n=4 PP and n=4 PV hepatocyte preparations from eight rat livers) and in dual-digitonin-pulse perfusion lysates (paired PP and PV samples from four rat livers). The sign "*" means that PV activity is significantly different (p<0.05, paired Student's t-test) from PP activity.
Figure 4-6  Immunoblot analysis of GST Ya and Yb2 from the cytosols derived from isolated PP and PV hepatocytes (n=4 PP and n=4 PV hepatocyte preparations from eight rat livers). Lanes 1-4 and 5-8, respectively denote samples from PP and PV hepatocyte cytosols. Proteins (5 µg) were separated by SDS-PAGE on 12% gels, transferred onto nitrocellulose and probed with antisera raised against rat GSTs (Biotrin). Detection was performed using the ECL method and immunoreactive bands were analyzed by densitometry (mean ± SD).
Figure 4-7  Immunoblot analysis of GST Ya and Yb2 from PP and PV dual-digitonin-pulse perfusion lysates (paired PP and PV samples from n=4 rat livers, #1 to #4). Lanes marked 1, 2, 3, and 4 denote paired PP and PV samples obtained from that particular rat liver. Proteins (5 µg) were separated by SDS-PAGE on 12% gel, transferred onto nitrocellulose and probed with antiserum raised against rat GSTs (Biotrin). Detection was performed using the ECL method and immunoreactive bands were analyzed by densitometry (mean ± SD). The symbol "*" denotes that the PV optical density is significantly different (p<0.05, paired Student’s t-test) from that of PP.
Figure 4-8 Immunoblot analysis of Mrp2/cMoat and CYP1A2 (a marker protein for PV abundance) from crude membranes (100,000g pellets) of isolated PP and PV hepatocytes (n=4 PP and n=4 PV hepatocyte preparations from eight rat livers). Lanes 1-4 and 5-8 indicate samples from PP and PV hepatocyte membrane fractions, respectively, of the various rat livers. Proteins (10 µg) were separated by SDS-PAGE on 7.5% (MRP2) and 9% (CYP1A2) gels, transferred onto nitrocellulose and probed with antibodies against rat Mrp2/cMoat (EAG15) and rat CYP1A2 (MAb 1-7-1). Detection was performed using the ECL method and immunoreactive bands were analyzed by densitometry (mean ± SD). The sign "*" denotes that PV optical density is significantly different (p<0.05, paired Student's t-test) from that of PP.
4.5 DISCUSSION

We had previously demonstrated utility of transport data from isolated rat hepatocytes and *in vitro* data on the spontaneous and enzymatic GSH conjugation in the prediction of GSH conjugation of EA in the whole liver (Tirona and Pang, 1999). It was concluded that various acinar factors on uptake, GSH availability, and distributions of the GSTs and GSH conjugate efflux systems among zonal cells could affect GSH conjugation. Although the identity of the EA transporter is uncertain and may be similar to that for bumetanide (Horz *et al.*, 1996), a sodium-independent saturable system that is inhabitable by organic anions was found to exist for EA uptake with rat hepatocytes (Tirona and Pang, 1999). The EA transporter appears to be homogeneously distributed within the liver acinus because there is no difference for EA uptake among the enriched PP and PV hepatocytes (Fig. 4-2). The kinetic parameters obtained from uptake experiments were similar among the zonal regions, and were not different to those obtained from hepatocytes prepared from all zonal regions (Tirona and Pang, 1999). Next to be considered is the aspect of zonal metabolism. In contrast to our anticipation of observing differences in metabolism at 200 μM EA (Tirona and Pang, 1999), initial rates of GSH conjugation within the PP and PV hepatocyte incubations were similar and closely resembled those for transport (Table 4-2). Moreover, GSH depletion rates were not different among PP and PV hepatocytes treated with EA (Figs. 4-3A and 4-4A), suggesting the absence of zonal influence by GSH. The EA-SG formation rates were, however, much lower than those found in the corresponding cytosolic fractions of PP and PV hepatocytes (Table 4-2), inferring strongly that hepatocyte EA uptake rate-limits GSH conjugation regardless of the zonal position along the sinusoidal plate.

The difference in GST activities within the cytosolic fractions of the PP and PV hepatocytes could have been revealed in the *in vitro* studies, except for the shallow or modest gradient in PV enrichment of GST activities towards EA and CDNB (Fig. 4-5). The results on GST activities coincided with immunodetectable GST Ya and Yb2 levels measured from cytosols of enriched PP and PV hepatocytes (Fig. 4-6). As demonstrated by others for CDNB (Kera *et al.*, 1987; Suolinna *et al.*, 1989), the PV enrichment of cytosolic GST activities was modest (1.2-1.6 fold). The PP and
PV lysates obtained by the dual-digitonin-pulse perfusion (Quistorff and Grunnet, 1987) offer an improved method for the study of metabolic heterogeneity in liver. With this technique, provision of the cellular contents of the most proximal or distal hepatocytes is accomplished by controlling the depth of digitonin penetration. These lysates proved to be more accurate in relating to PP and PV activities, and showed 2- to 3-fold PV predominance in GST activity towards EA and CDNB (Fig. 4-5) and complement corresponding changes in immunoreactive GSTs - Ya and Yb2 in the lysate (Fig. 4-7). These results, along with those from isolated PP and PV hepatocytes, confirm that acinar gradients in GST activity towards EA and CDNB exist, and the gradients are relatively shallow. Comparable observations were reported for CDNB GST activities (Kera et al., 1987) and GST protein contents in PP and PV lysates (Lindros et al., 1998). The slightly lower GST activity in PP lysate as compared with PV lysate (155 ± 44 and 364 ± 42 nmol/min/mg cytosolic protein respectively, Fig. 4-6A) towards EA would not rate-limit EA elimination because the transport activity is much lower.

Our metabolic data demonstrate that limitations existed in the intact, isolated PP and PV hepatocyte system for the study of functional metabolic heterogeneity. In examination of the PP marker enzyme ALT, the PP/PV activity ratio (~1.8) normally obtained was considerably lower than that (~7.5) in lysates (Table 4-1). This difference underscores the fact that enriched zonal hepatocytes are harvested from approximately half of the sinusoidal length and are inevitably cross-contaminated to a higher degree (especially from midzonal regions) than for lysates which are derived from only a small population of hepatocytes at the most distal and proximal acinar regions. Therefore, depending on the shape and steepness of the zonal distribution of enzymatic activities, difficulties persist to identify shallow gradients on metabolic activities, as found in this study. However, the enrichment in PV:PP ratio of GS was not much improved with the lysate over the hepatocytes because the distribution of GS is mostly confined to the last cells around the hepatic venules (Burger et al., 1989) and will not be perturbed much by the contamination of cells from other zonal regions in which GS was absent. Analogously, we cannot exclude the possibility that a shallow gradient exists for EA transporters although we did not observe a difference in EA uptake.
among the zonal cells. There is also the possibility that the contents of non-parenchymal cells are sampled during dual-digitonin-pulse perfusion and this may also contribute to the differences seen in the metabolic activities observed in lysates and hepatocyte cytosols.

The aspects of accumulation and efflux of EA-SG were further addressed. Because product inhibition of the GSTs by EA-SG is known to exist (Ploemen et al., 1990, 1993), Mrp2 may play a role in GSH conjugation due to the removal of EA-SG at the canalicular membrane. We have shown that 90% of the formed EA-SG was rapidly excreted into bile of the rat liver preparation (Tirona and Pang, 1999), suggesting that canalicular transport predominates over cellular efflux mediated perhaps by the Mrp family proteins present on the lateral or basolateral membranes (Zaman et al., 1997). Although human MRP2 was found to transport EA-SG (Evers et al., 1998), rat Mrp isoforms have not been directly shown to transport EA-SG. However, there appears to be a close substrate specificity between the human and rat orthologs (Keppler et al., 1998), and ATP-dependent uptake of the typical Mrp2 substrate, dinitrophenyl-GSH conjugate, into rat canalicular membrane vesicles was almost completely inhibited by EA-SG (Ballatori and Truong, 1995). It is hence reasonable to assume that Mrp2 is primarily responsible for the efflux of EA-SG by hepatocytes. From our studies, it was noted that the initial EA-SG efflux rate among PP and PV hepatocytes were similar (Table 4-2) suggesting homogeneity in Mrp2 function within the liver acinus. The observation is consistent with the lack of zonal differences in the levels of immunoreactive Mrp2 protein as determined by immunoblotting (Fig. 4-8) and acinar homogeneity in MRP2 described by Kool et al. (1999) in human liver using an immunohistochemical technique. Recently, Morrow et al. (1998) demonstrated that the combined effect of enhanced expression of GST P1-1 and MRP1 in MCF7 breast carcinoma cells conferred resistance to the cytotoxic effects of EA. Elevated expression of either GST P1-1 or MRP1 alone did not cause significant resistance to toxicity. Given the dual presence of GSTs and Mrp2 in hepatocytes throughout the acinus, all hepatocytes contain comparable cytoprotective defenses against exposure to electrophiles such as EA.
The greater GSH conjugation over the efflux activity usually suggests an accumulation of EA-SG. However, this was not observed. EA-SG accumulated only transiently at the onset of incubation within isolated zonal hepatocytes due to the cessation of EA-SG formation as GSH became depleted, and due to the rapid efflux into extracellular medium (Figs. 4-3 and 4-4). It remained plausible that product inhibition of the GSTs ensued during the early time period. The cessation of EA-SG formation and rapid efflux brought about the decline in intracellular concentrations. However, EA-SG persisted within the cell after 20 min, albeit at a low and constant level. This observation is consistent with findings on the redistribution or internalization of Mrp2 from the cell surface to endosomes and lysosomes shortly after isolation of the hepatocytes (Oude Elferink et al., 1993; Roelofsen et al., 1995), although in the native intact liver, the majority of Mrp2 protein is localized on the apical membrane. Therefore, intracellular EA-SG remaining beyond 20 min of incubation most likely signifies that the conjugate is sequestered within the intracellular vesicles (Fig. 4-1). Indeed, the fitting exercise supports the above conjecture. Absence of the sequestered pool or intracellular vesicles inevitably led to poorer fits to the data.

In conclusion, we demonstrated that the GST activities responsible for the GSH conjugation of EA were high, and a relatively shallow and increasing acinar gradient existed in rat liver. Despite this zonal heterogeneity in metabolic activity, uptake was uniform throughout the acinus, and this, together with low GSH levels, rate-limited the GSH conjugation of EA. The major efflux system for EA-SG occurs most likely via Mrp2, and acinar homogeneity in Mrp2 function was observed. However, the present studies only dealt with uptake and metabolism in hepatocytes. In extrapolating data to the whole liver, one must be cognizant of the impact of non-parenchymal cells (Kupffer, Ito and endothelial cells) which also contain GSTs, albeit at reduced levels (Steinberg et al., 1989, Parola et al., 1993, Lee et al., 1994). The presence of other cell types and associated heterogeneities might further affect the hepatic GSH conjugation of EA.
4.6 STATEMENT OF THE SIGNIFICANCE OF CHAPTER 4

In this chapter we measured the uptake and metabolism of EA by isolated enriched PP and PV rat hepatocytes and directly determine that uptake rate-limits the GSH conjugation of EA. Moreover, the uptake capacity for EA by PP and PV hepatocytes is similar although a shallow and increasing gradient in enzymatic GSH conjugation exists within the liver acini. Furthermore, we demonstrate for the first time that Mrp2/cMoat function is homogeneously distributed in rat liver using EA-SG as a probe substrate.

4.7 ACKNOWLEDGMENT

We thank Dr. Dietrich Keppler (Deutsches Krebsforschungszentrum, Heidelberg Germany) and Dr. Harry V. Gelboin (National Cancer Institute, National Institutes of Health, Bethesda, MD, USA) and for providing us with the antibodies towards Mrp2/cMoat and CYP1A, respectively, and Dr. J.H.T.M. Ploemen (TNO, Ziest, The Netherlands) for supplying the [14C]EA.
CHAPTER 5

DISTRIBUTED-IN-SPACE MODELING OF GLUTATHIONE CONJUGATION OF ETHACRYNIC ACID IN PERFUSED RAT LIVER

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5.1 ABSTRACT

Inasmuch as \textit{in vitro} uptake and metabolic studies with enriched periportal (PP) and perivenous (PV) hepatocytes revealed rate-limitation by uptake and not metabolism in the overall glutathione (GSH) conjugation of ethacrynic acid (EA) (Tirona et al., 1999), data obtained from the perfused rat liver preparation (Tirona and Pang, 1999) were re-examined to explain the observation of the uncharacteristically high extraction ratio of EA with high plasma protein binding; the aberrant observation could have been attributed to the zonal occurrence of GSH conjugation. To investigate further the effect of plasma protein binding and distributed-in-space modeling on EA removal, single pass liver perfusion was conducted in the presence and absence of albumin (BSA) in erythrocyte-free medium; the concentration of \( \sim 44 \mu M \) was chosen because hepatic GSH depletion existed with BSA in perfusate, and an even greater perturbation was expected with absence of BSA. Indeed, the moderately high, steady state extraction ratio of EA \( (E_s \sim 0.64) \) that existed between 10 to 45 min of perfusion with 1\% BSA increased to \( \sim 0.99 \) during the first 20 min of perfusion in absence of BSA, followed by a decline in the value. These results corresponded to the greater rate of hepatic GSH consumption/depletion in absence of BSA. However, attempts on histochemical measurements of hepatic GSH in the acinus with mercury orange staining failed; there was rapid loss of GSH upon tissue fixation with paraformaldehyde and poor morphology of the liver with un-fixed frozen sections. Common models of hepatic drug elimination - the “parallel tube” and the “dispersion models” - were more consistent in relating previously obtained \textit{in vitro} data to the GSH conjugation of EA in absence of BSA than the “well-stirred model”. But all of the models failed to predict the data in the presence of BSA because of their inherent assumptions on equilibrative binding and transport of the unbound drug only. Hence, a series compartment model which encompassed information on acinar uptake and metabolism of EA in (PP) and perivenous (PV) hepatocytes was developed to predict the rates of GSH conjugation and zonal GSH depletion. Whereas this model was consistent with observations derived from BSA-free perfusion, the model required unrealistically high membrane permeability and binding disequilibrium constants of EA to albumin (extremely rapid on and off rate constants) for predicting the observations on EA with 1\% BSA. In both instances, the series model predicted the spatial depletion of GSH, occurring first in
PP (upstream) hepatocytes then eventually and to a lesser degree, the PV zone, and conferred time-dependence of the varying rate-limiting steps in GSH conjugation among hepatocytes. However, acinar GSH measurements would have greatly substantiated the validity of the temporal-spatial modeling.

5.1 INTRODUCTION

It is becoming apparent that hepatic drug removal kinetics are governed by both uptake and metabolism/excretion and occur in a distributed-in-space fashion (Goresky et al., 1998; Pang et al., 1998). For the bimolecular glutathione (GSH) conjugation reaction, the acinar availability of GSH is another important variable that can impact on the local GSH conjugation rate and ultimately, the whole organ reaction rate. The interplay of the factors affecting hepatic GSH conjugation of xenobiotics has been seldom modeled. In these instances, hepatic GSH conjugation kinetics of ethylene dichloride (D'Souza et al., 1988), acetaminophen (Chen and Gillette, 1988), butadiene monoxide (Johanson and Filser, 1993), styrene oxide (Csanady et al., 1994) and dichloroethylene (El-Masri et al., 1996) have been successfully modeled with use of physiologically based modeling approaches. A deficiency of these earlier models, however, remains because they do not take into account the acinar (spatial) factors on the cofactor, glutathione (GSH) or enzyme(s), the glutathione S-transferases (GSTs) or other bioactivation enzymes as considered by Chiba and Pang (1995).

In a previous investigation, we characterized the concentration-dependent GSH conjugation kinetics of the model electrophile, ethacrynic acid (EA), in the perfused rat liver. Together with data on hepatocyte uptake and in vitro metabolism, the results suggest that cellular influx rate-limited the hepatic elimination of EA. However, a relatively high steady-state hepatic extraction (0.5-0.8) prevailed despite extensive binding to BSA or bovine serum albumin (unbound fraction $f_u$ of 0.02-0.06) (Tirona and Pang, 1999). Attempts to model the protein binding effects on EA removal by a "well-stirred" physiologic model that assumed sole uptake of unbound ligand and rapid equilibrative binding were unsuccessful. Rather, adequate fits to the data were accomplished only when the total plasma EA concentration (sum of free and bound) was capable of undergoing hepatic uptake.
Because acinar transport, enzymic distribution of GSTs and GSH availability could influence GSH conjugation in whole liver and clearance estimates (Pang, 1995; Kwon and Morris, 1997), the importance of these variables in relation to GSH conjugation was recently studied in isolated enriched periportal (PP) and perivenous (PV) rat hepatocytes (Tirona et al., 1999). There was no transport heterogeneity and there was a lack of difference in the rate of GSH conjugation among zonal cells even with an increasing PP to PV gradient in GST activities towards EA. The rate of GSH conjugate (EA-SG) formation with intact zonal hepatocytes was similar to uptake rate, but was lower than the rate of GSH conjugation in vitro, suggesting that transport and not enzymatic activity rate-limited the overall removal of EA in hepatocytes. With this additional information, it becomes possible to refine previous kinetic models of hepatic EA disposition in a manner consistent with the concept of distributed-in-space processing.

The purpose of this report was to develop a distributed-in-space model to comprehensively view protein binding/debinding, transport, GST and GSH on hepatic EA elimination occurring within hepatocytes along the direction of flow. To this end, we monitored EA clearance/GSH conjugation rates and GSH depletion in biopsied samples of rat livers that were perfused with BSA-containing or BSA-free media at a concentration (44 µM) previously shown to result in GSH depletion (Tirona and Pang, 1999). It was envisioned that more substrate would enter upstream (PP) hepatocytes in the absence of binding protein, thereby resulting in a faster and more localized depletion of GSH. By contrast, protein binding of EA would buffer substrate uptake by PP hepatocytes, sparing the substrate such that it would now reach the more distal (PV) hepatocytes. We expected that the difference in protein binding of EA would result in experimental perturbations of acinar GSH conjugation that may lead to the development of a kinetic model for description of GSH conjugation. Additionally, the adequacies of several common models of hepatic clearance - the “well-stirred”, “parallel tube” and “dispersion models” - were appraised in their ability to predict observations of the perfused liver from scale-up of in vitro data. Furthermore, a series compartment model which incorporated acinar transport, GST, and GSH, was developed to describe the hepatic disposition of EA.
5.3 METHODS

5.3.1 Chemicals. EA, BSA, GSH and mercury orange were obtained from Sigma (St. Louis, MO). EA-SG and other thiol metabolites were synthesized as described previously (Tirona and Pang, 1999). All other reagents were of the highest available grade.

5.3.2 Rat Liver Perfusion. Male Sprague-Dawley rats (Charles River, St. Constant, PQ) served as liver (wt. 11.4 ± 1.4 g) donors for in situ perfusion as detailed by Pang et al. (1988). Because erythrocytes are known to metabolize EA (Tirona and Pang, 1999), livers were perfused in a single pass fashion at a flow rate of 25 ml/min with erythrocyte-free Krebs-Henseleit buffer (pH 7.4, 37°C) containing 300 mg% glucose without EA for 10 min of equilibration followed by perfusate containing EA for the next 45 min. In one set of studies, BSA (1%) was present, whereas in the second set of studies, BSA was absent. The concentration of ~44 μM EA was chosen because previous perfusions at this concentration had produced an observable depletion of hepatic GSH over the time course of 45-min perfusion. It was expected that perfusion without BSA would cause a greater disturbance in hepatic GSH. In a third set of control experiments, livers were perfused with drug-free, BSA containing medium. At regular intervals, input and venous output perfusate as well as bile were sampled for drug and metabolite assays. Prior to drug administration and at 15 min intervals, lobes of the liver (papillary process, right lateral, caudate and left lateral lobes) were ligated with silk sutures (to prevent perfusate leakage) and removed for GSH analysis. These biopsies resulted in excision of approximately 17% of the total liver weight and did not have any deleterious effects on gross liver function as assessed by bile flow. One section of liver was immersed into OCT compound and rapidly frozen in a dry ice/ethanol bath then stored at -70°C. The remaining liver section was used immediately for the biochemical determination of GSH.

5.3.3 Analyses. Perfusate and bile samples were acid-stabilized and processed as previously described (Tirona and Pang, 1999). EA and its metabolites were measured by high-performance liquid chromatography (HPLC) (Tirona and Pang, 1999). GSH in liver biopsies was determined by the HPLC method of Fariss and Reed (1985).
5.3.4 **GSH Histochemistry.** In one set of experiments, acinar GSH concentrations were assessed by the method of Asghar et al. (1975), with slight modifications. The frozen liver biopsy sample was sectioned (~10 μm) on a cryostat (-20° C) and placed on a glass slide. The section was thawed for a few seconds and immersed into an ice-cold solution of 50 μM mercury orange in toluene for 1 min. The slide was rinsed three times with acetone and then cleared with toluene. After mounting with Permount (Fisher Scientific), the slide was viewed with a fluorescence microscope (Zeiss) fitted with a 100 watt mercury lamp, BP 450-490 nm excitation filter and a FT 510 beam splitter (Sherman and Fisher, 1986) with observations made through a BP 520-560 nm barrier filter. For other experiments, the histochemical measurement of GSH was performed according to Forkert and Moussa (1989). At various times following EA perfusion, the liver was perfusion fixed with ice-cold paraformaldehyde (4%) in 100 mM potassium phosphate (pH 7.4) for 5 min. Pieces of fixed liver were then cryopreserved overnight in 1% paraformaldehyde buffer containing 30% sucrose at 4° C. The tissues were then frozen with OCT compound and stored at -70° C until sectioning. The staining procedures for fixed tissues were identical to that described above for unfixed tissues.

5.3.5 **Correlation of Data to Hepatic Clearance Models.** The ability of several models of hepatic drug clearances - the “well-stirred” model (Rowland et al., 1973), “parallel tube” model (Winkler et al., 1973) and “dispersion model” (Roberts and Rowland, 1986) was appraised with respect to predicting the hepatic GSH conjugation of EA based on the scale-up of *in vitro* data. Data obtained previously on EA hepatocyte uptake and equilibrium binding to BSA were used (Tirona and Pang, 1999). The following assumptions were made:

1. The elimination of EA in the rat liver is rate-limited by uptake; the assumption is reasonable because data in hepatocytes support the claim (Tirona et al., 1999).

2. The uptake clearance of the whole liver is reflected by the transport capacity of isolated hepatocytes upon scale-up based on cellularity.

3. Only unbound EA is capable of membrane transport; the unbound drug and bound drug are in rapid equilibrium.
The role of GSH in the bimolecular GSH conjugation reaction is unimportant at steady-state, because a pseudo first-order reaction scheme is assumed, and the maximal values of clearance and extraction ratios are obtained.

Based on assumption (2), the uptake clearance for the whole liver is estimated by

$$PS_{in} = \frac{V_{\text{uptake max}}}{K_{\text{uptake}} + [EA_{\text{unbound}}]} + P_{\text{diff}} \left( \frac{\alpha}{\beta} \right)$$  \hspace{1cm} (5-1)

where, $V_{\text{uptake max}}$, $K_{\text{uptake}}$, and $P_{\text{diff}}$ are parameters describing the maximum hepatocyte uptake rate, the Michaelis-Menten constant for saturable uptake, and the linear uptake clearance, respectively, whereas $[EA_{\text{unbound}}]$ is the unbound EA concentration in the sinusoids. The scaling factors $\alpha$ and $\beta$ are $1.25 \times 10^8$ cells/g liver and $1 \times 10^6$ cells/mg protein.

The steady state extraction ratio ($E_u$) for EA may be predicted for a given unbound plasma fraction ($f_u$) using the "well-stirred", "parallel-tube" and "dispersion" models.

For the "well-stirred" model, the highest value of the steady state extraction ratio $E_{u,ss}$ is (Gillette and Pang, 1977),

$$E_{u,ss} = \frac{f_u CL_{\text{int}} PS_{in}}{Q PS_{out} + f_u CL_{\text{int}} PS_{in} + Q CL_{\text{int}}}$$  \hspace{1cm} (5-2)

where $PS_{out}$ and $CL_{\text{int}}$ are the efflux transport clearance and the intrinsic (metabolic) clearance for GSH conjugation of EA, respectively, under pseudo first-order conditions. When uptake-limited removal exists (i.e. $PS_{in} >> CL_{\text{int}}$ and $CL_{\text{int}} >> PS_{out}$), the equation simplifies to

$$E_{u,ss} = \frac{f_u PS_{in}}{f_u PS_{in} + Q}$$  \hspace{1cm} (5-2A)

For the "parallel-tube" model, $E_u$ is given by

$$E_{u,ss} = 1 - e^{-\left( f_u CL_{\text{int}} PS_{in} \right) / \left( Q CL_{\text{int}} + PS_{out} \right)}$$  \hspace{1cm} (5-3)

and with uptake-limited removal, the equation simplifies to

$$E_{u,ss} = 1 - e^{-\left( f_u PS_u / Q \right)}$$  \hspace{1cm} (5-3A)

For the "dispersion" model, $E_u$ is given by
In Eq. 5-4, $D_N$ is the dispersion number relating to the degree of dispersion of the system and is assigned a value of 0.15 (Tirona et al., 1998), whereas $R_N$ is

$$R_N = \frac{f_u P_{SA} \cdot CL_{int}}{Q (P_{Sout} + CL_{int})}$$

(5-5)

When uptake-limited removal exists, Eq. 5-5 is reduced to

$$R_N = \frac{f_u P_{SA}}{Q}$$

(5-5A)

and Eq. 5-4 becomes,

$$E_{ss} = 1 - e^{-\left(\frac{1}{2D_N}\right)^n}$$

(5-4A)

5.3.6 Physiological Modeling.

5.3.6.1 BSA-Free Perfusion. The disposition of EA during protein-free perfusion was analyzed using an elaboration of a previous well-stirred physiologic model (Tirona and Pang, 1999) that was converted to a series compartment scheme (Fig. 5-1) not unlike that described for salicylamide metabolism (Tirona and Pang, 1996). In the present model, an expansion of three (that denotes zones 1, 2, and 3) to six zonal units was utilized because the present scheme allowed for higher spatial resolution for metabolic or transport zonation and an increase in the efficiency of hepatic drug removal. These units are connected sequentially along the direction of flow, with each unit being subcompartmentalized to its sinusoidal, cellular, and bile compartments with parameters being
Figure 5-1 “Series compartment” model for hepatic EA disposition during protein-free perfusion. The liver is composed of 6 zonal units, each comprising a sinusoid and cellular compartments. EA uptake is by saturable (denoted by $V_{\text{max}}^{\text{uptake}}$ and $K_{m}^{\text{uptake}}$) and nonsaturable ($P_{\text{diff}}$) processes. Glutathione conjugation of EA proceeds enzymatically catalyzed by the glutathione S-transferases (GSTs, with kinetic constants $K_{m}^{\text{EA}}$, $K_{m}^{\text{GSH}}$, and $V_{\text{max}}^{\text{metab}}$) and nonenzymatically (with the bimolecular constant, $k_2$). The intracellular concentration of GSH was described by its synthesis ($k_{\text{syn}}(\text{GSH})$) and degradation ($CL_{\text{deg}}(\text{GSH})$). The EA-SG formed undergoes net biliary excretion ($CL_{b}(\text{EA-SG})$), or undergoes net sinusoidal efflux ($P_{\text{diff}}(\text{EA-SG})$). EA-SG efflux occurs into a “Bile Compartment” and biliary excretion out of this compartment is governed by the bile flow rate ($Q_{\text{bile}}$). Acinar heterogeneity in enzymatic GSH conjugation is shown in the scheme below. An increasing PP to PV gradient in $V_{\text{max}}^{\text{metab}}$ that was consistent with previous in vitro findings (Tirona et al., 1999) was optimized to fit the observed data.

The initial cellular GSH concentration ([GSH]$^{0}$) was set according to the measured value of hepatic GSH acquired by biopsy prior to perfusion of the liver with EA. In view of the absence of appreciable GSH synthesis in hepatocytes incubated in a medium lacking amino acid precursors (Tirona et al., 1999), we have allocated values for GSH synthesis and its degradation clearance ($K_{\text{syn}}$ and $CL_{\text{deg}}(\text{GSH})$, respectively) to zero. Based on the lack of definitive data in the literature on the zonal concentrations of GSH and due to observations that GSH levels in PP and PV hepatocytes were similar (Tirona et al., 1999), we have adopted a homogeneous distribution in the initial GSH contents for all cellular compartments.
The differential equations governing hepatic EA kinetics during BSA-free perfusion are given in the Appendix, and the parameters are shown in Table 5-1.

5.3.6.2 BSA Perfusion. In the presence of BSA, changes with protein binding exist only in the sinusoidal compartments (Fig. 5-2); the kinetic events dictating transport, metabolism, and excretion in the cellular and bile compartments remain identical as those described previously (Fig. 5-1). Because clearance models assume rapid binding equilibrium in the sinusoids and are incapable of predicting EA elimination in the presence of BSA (see Results), the series compartment model was modified to incorporate sinusoidal protein binding disequilibrium. In this variation, EA exists in the sinusoidal compartments as an unbound species or one bound to high (superscripted as “A”) and low (superscripted as “B”) affinity binding sites on BSA, and only unbound drug is capable of transport into the cellular compartments (Fig. 5-2). Binding and debinding of EA to BSA is described by “on” and “off” rate constants, \( k_{on} \) and \( k_{off} \), respectively. The additional differential equations governing EA binding and debinding in the sinusoids during BSA perfusion experiments are given in the Appendix, and the additional binding parameters are shown in Table 5-1. All other differential equations for the cellular and Bile compartments are similar to the protein-free condition.

5.3.6.3 The Bile Compartment. The “Bile” compartment was added and assigned a volume of 0.02 ml, similar to that found by Reichen and Paumgartner (1980). The rates of bile flow, however, were non-constant with time. The bile flow could be fitted empirically to a sum of exponentials,

\[
Q_{bile} = Ae^{-\alpha t} + Be^{-\beta t}
\]

(5-6)

with the program, SCIENTIST (Micromath Scientific Software, Salt Lake City, UT) (Fig. 5-3). This yielded the parameter values of -0.0215 and 0.0289 ml/min for A and B, respectively, and 0.183 and 0.0123 min^{-1} for \( \alpha \) and \( \beta \), respectively, for the experiments without BSA. The values for A and B were -0.0154 and 0.264 ml/min, respectively, and \( \alpha \) and \( \beta \) were 0.023 and 0.006 min^{-1}, respectively, for the experiments with BSA (Fig. 5-3). The bile flow rates were generally similar in either absence or presence of albumin.
### Table 5-1. Parameters for the series-compartment model of EA disposition in perfused liver.

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<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
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<tbody>
<tr>
<td>Q</td>
<td>Perfusate flow rate</td>
<td>25 ml/min&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Q&lt;sub&gt;bile&lt;/sub&gt;</td>
<td>Bile flow rate</td>
<td>fitted with Eq. 5-6</td>
</tr>
<tr>
<td>V&lt;sub&gt;p&lt;/sub&gt;</td>
<td>Volume of each sinusoid compartment</td>
<td>0.067 ml&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V&lt;sub&gt;c&lt;/sub&gt;</td>
<td>Volume of each cell compartment</td>
<td>0.107 ml&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>V&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Volume of the Bile compartment</td>
<td>0.02 ml&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>[GSH]&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Initial hepatocyte GSH concentration</td>
<td>8.6 mM&lt;sup&gt;a&lt;/sup&gt;</td>
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**Uptake**
- \( K_{m,\text{uptake}} \): Michaelis-Menten constant for EA uptake | 57 μM<sup>a</sup> |
- \( V_{\text{uptake}}^{\text{max}} \): Maximum facilitated uptake velocity for EA for each compartment | For -BSA 1.0 nmol/min<sup>a</sup> |
- \( P_{\text{diff}} \): Bi-directional transmembrane clearance for EA for each compartment | For +BSA 50 nmol/min<sup>a</sup> |

**Metabolism**
- \( k_{z} \): Second-order spontaneous GSH conjugation constant | 126 μM<sup>-1 min</sup><sup>a</sup> |
- \( K_{m,\text{GSH}} \): Michaelis-Menten constant for GSH in enzymatic reaction | 94 μM<sup>a</sup> |
- \( K_{m,\text{EA}} \): Michaelis-Menten constant for EA in enzymatic reaction | 1.2 mM<sup>a</sup> |
- \( V_{\text{max}}^{\text{max}} \): Maximum enzymatic GSH conjugation rate for each compartment | see Fig. 5-1 for zonal gradient |

**Metabolite Efflux**
- \( Cl_{\text{EA-SG}}^{\text{EA}} \): Net EA-SG sinusoidal efflux clearance for each compartment | 0.013 ml/min<sup>a</sup> |
- \( Cl_{\text{EA-SG}}^{\text{b}} \): Net EA-SG biliary efflux clearance for each compartment | 0.2 ml/min<sup>a</sup> |

**Plasma Protein Binding**
- \( B_{\text{max}}^{\text{h}} \): Maximum concentration of high affinity EA binding site on BSA | 187 μM<sup>a</sup> |
- \( B_{\text{max}}^{l} \): Maximum concentration of low affinity EA binding site on BSA | 694 μM<sup>a</sup> |
- \( k_{a,b}^{\text{h}} \): Association rate constant for high affinity EA binding site on BSA | 4000 μM<sup>-1 min</sup><sup>a</sup> |
- \( k_{a,b}^{l} \): Association rate constant for low affinity EA binding site on BSA | 4000 μM<sup>-1 min</sup><sup>a</sup> |
- \( k_{d,b}^{\text{h}} \): Dissociation rate constant for high affinity EA binding site on BSA | 10400 min<sup>a</sup> |
- \( k_{d,b}^{l} \): Dissociation rate constant for low affinity EA binding site on BSA | 400000 min<sup>a</sup> |

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<sup>a</sup> From Tirona and Pang (1999)
<sup>b</sup> Assumed based on measured initial hepatic GSH content
<sup>c</sup> Experimental condition
<sup>d</sup> From in vitro equilibrium binding data (Tirona and Pang, 1999)
<sup>e</sup> Optimized value
<sup>f</sup> From Reichen and Paunggartner (1980)
Scheme for protein binding disequilibrium of EA in the i\textsuperscript{th} sinusoidal compartment for the BSA perfusion experiments. EA exits as an unbound species (\(EA_{\text{unbound}}\)) or bound to high (\(EA_{\text{boundA}}\)) and low (\(EA_{\text{boundB}}\)) affinity sites on BSA. Binding/debinding rates are functions of \(k_{\text{on}}\) and \(k_{\text{off}}\) for each of the sites. Binding constants are constrained to be consistent with \textit{in vitro} equilibrium binding studies (Tirona and Pang, 1999). Unbound EA is transported into the cellular compartments and is described by saturable (unidirectional) and linear (bidirectional) processes.

5.3.7 Simulations

Because the recoveries of drug and metabolites escaping the liver were \(\sim 90\%\) of the EA input (see results), the data were scaled up proportionately to 100\%. Simulations with the series compartment model were performed with the program, SCIENTIST with differential equations shown in the Appendix. The magnitudes of the enzymatic GSH conjugation activity within each zone, \(V_{\text{metab}}\), were optimized by trial-and-error to best conform to the mean experimental observations. All other parameters were assigned based on \textit{in vitro} findings with appropriate scale-up.

Additionally, for the studies conducted with BSA, values for the binding rate constants were constrained during the simulation to be consistent with results of previous equilibrium binding experiments (Tirona and Pang, 1999), namely, the ratio of \(k_{\text{offA}}/k_{\text{offA}}\) was set equal to \(K^A_d\) or 2.6 \(\mu\)M.
for the high affinity binding constant on BSA, and the total number of high affinity binding sites ($B_{\text{max}}^A$ or $n[P]$) was 187 µM. Analogously, the low affinity binding site (B) was arbitrarily given a $K_D^B$ value of 100 µM and the $B_{\text{max}}^B$ value was set as 694 µM to maintain consistency with the in vitro value for the linear binding site ($k_L=6.9$) (Tirona and Pang, 1999). Again, the ratio of $k_{\text{vitr}}/k_{\text{unb}}$ was constrained to equal $K_D^B$. Hence, with an EA input concentration of 44 µM, 88% of the drug is initially bound to the high affinity site (A), 10% bound to the low affinity site (B), and only 2% exists as unbound drug in plasma.

5.3.8 Data analysis. All data are presented as mean ± S.D. The Student’s $t$-test was performed for the comparison of means, with the significance level set at 0.05.

**Fig. 5-3** Observed bile flow rates during EA (44 µM) perfusions in the presence (●) and absence (○) of BSA and in control (■, no EA). The lines for EA perfusions are based on best fit of data with the equation $Q_{\text{bile}} = Ae^{-ut} + Be^{-bt}$. Data are presented as mean ± S.D.
5.4 RESULTS

5.4.1 Effect of protein binding on hepatic EA disposition.

5.4.1.1 BSA-Free Studies. With administration of EA (44 ± 4 μM or 102 ± 14 nmol/min/g liver, n=3) in absence of BSA to the rat liver preparation, an almost complete extraction of EA (Eₐ = 0.99 ± 0.01, p<0.05) was observed during the first 20 min of perfusion with 44 ± 2 μM of EA (or 97 ± 10 nmol/min/g liver, n=3) (Fig. 5-4A). This was immediately followed by a decline of steady-state extraction ratio to ~ 0.72 by the end of the experiment. The loss of EA was accompanied by the appearance of EA-SG, mostly into bile and minimally in venous output (Fig. 5-4B). EA-SG was the major metabolite detected in bile, and only minor levels of cleavage thiol metabolites were observed. The high rate of biliary EA-SG excretion (~80 nmol/min/g liver) remained constant until the last 5 min of the experiment (Fig. 5-4B). Biliary excretion of EA-SG amounted to 94 ± 3 % of the total EA-SG output, and the overall recovery of EA and metabolites represented 89 ± 7 % of the input. Loss of GSH was dramatic in absence of BSA (106 ± 24 nmol/min/g liver), decreasing from an initial value of 6.3 μmol/g liver to 1.5 μmol/g liver at the end of the experiment (Fig. 5-4C). The level of GSH at the end of the perfusion study was significantly different from those of the control livers (p<0.05) whose concentration remained quite constant with perfusion (Fig. 5-4C).

5.4.1.2 BSA-Studies. With administration of EA (44 ± 4 μM or 102 ± 14 nmol/min/g liver, n=3) in 1% BSA perfusate to the rat liver, a constant extraction ratio (Eₐ of 0.64 ± 0.04) was observed from 5 min onwards, because the hepatic venous output of EA remained constant (Fig. 5-4A). The Eₛ value was substantially lower than that in absence of BSA, but was similar to that observed previously for rat livers perfused at comparable EA concentrations in 1% BSA (Tirona and Pang, 1999). The biliary excretion of EA-SG was again lower than that in the BSA-free media (~80 nmol/min/g liver, p<0.05; Fig. 5-4B). The excretion rates approached steady-state by 10 min and remained at ~ 60 nmol/min/g liver until completion of the experiment. Biliary excretion of EA-SG amounted to 96 ± 3 % of the total EA-SG output, and the overall recovery of EA and metabolites represented 91 ± 15 % of the input. The rate of GSH depletion was less severe for the perfusions with BSA (57 ± 21 nmol/min/g liver, vs 106 ± 24 nmol/min/g liver without BSA, p<0.05) (Fig. 5-4C). By the end of the experiment, the hepatic GSH content remaining (~ 3.5 μmol/g liver) was
higher than those in livers perfused in absence of BSA (\(\sim 1.5 \, \mu\text{mol/g liver}\)) although no statistical difference was detected \((p = 0.11)\). Again, the level of GSH was significantly different from that of the control livers \((p<0.05)\) whose concentration remained quite constant with perfusion (Fig. 5-4C).

5.4.2 Prediction of Hepatic Clearance Models on EA Conjugation. The uptake clearance \((PS_{in})\) estimated for the whole organ (based on Eq. 5-1 and EA concentration of 44 \(\mu\text{M}\)) was 91 ml/min/10 g liver, a value which is \(\sim 3.6\) times the perfusion flow rate (25 ml/min). Given the unbound fraction of 1 and 0.02 for EA in the absence and presence of BSA respectively, the maximal \(E_{in}\), based on pseudo first-order conditions according to the “well-stirred”, “parallel-tube” and “dispersion” models are summarized in Table 5-2. For livers perfused with BSA, all models severely underestimated the hepatic extraction of EA by 10-fold. However for perfusions without BSA, the “parallel-tube” and “dispersion” models provided relatively accurate predictions; the “well-stirred” model rendered an underestimate of the extraction ratio of EA.

With the scale-up procedure described within, one must be cautious of the requirement for kinetic linearity. This procedure utilizes a non-linear equation (Eq. 5-1) for the estimation of whole organ \(PS_{in}\) that is dependent on the assignment of \([EA_{unbound}]\). For calculations, we used a concentration of 44 \(\mu\text{M}\), which would be the maximum concentration to which hepatocytes would be exposed. Because on average, hepatocytes encounter unbound EA concentrations lower than the input level (especially when BSA is present), the “mean” \(PS_{in}\) value would be larger and would reach a maximum value of 115 ml/min/10g liver in the tracer condition (Tirona and Pang, 1999). Therefore the extraction ratios estimated by these models are slightly underpredicted.
Figure 5-4

Results of liver perfusion studies of EA with (n=3) and without (n=3) BSA. Hepatic venous output rate of EA-BSA (■) and arterial output rate of EA-BSA (○). Data are presented as means ± S.D. Statistically different from control experiments (unpaired t-test).
Table 5-2. Summary of predicted and observed steady-state extraction ratios for EA (~44 μM) perfusions in the presence (n=3) and absence (n=3) of BSA.

<table>
<thead>
<tr>
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<th>Steady-State Extraction Ratio (Eₜₜ)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td></td>
<td>Well-Stirred</td>
</tr>
<tr>
<td>+ BSA</td>
<td>0.64 ± 0.04</td>
</tr>
<tr>
<td>- BSA</td>
<td>0.99 ± 0.01</td>
</tr>
</tbody>
</table>

a with sinusoidal binding disequilibrium

5.4.3. Physiologic Modeling.

5.4.3.1 BSA-Free Perfusions. Upon optimization of the zonal gradient for enzymatic GSH conjugation (Fig. 5-1) and correction for the observed mass imbalance, excellent correspondence was observed in the simulated hepatic output of EA and EA-SG and the experimental data with protein-free perfusions (Fig. 5-5A). Similarly, the model closely matched the time-dependent decline in total hepatic GSH concentrations (Fig. 5-5B).

Given the close agreement of the model with the input/output data, the time-dependent acinar gradient in EA concentrations was simulated (Fig. 5-7A). During the first 20 min, EA concentrations along the porto-venous axis declined exponentially such that only a small fraction of the input drug escaped the liver, reflecting high hepatic extraction. With increasing perfusion time, however, the shape of the concentration gradient changed, and the extraction ratio decreased. It can be seen that near the completion of the experiment, the contribution of the first 3 compartments to the removal of EA became minimal and much attenuated, and the sinusoidal concentration of EA was not greatly decreased during the traverse of the upstream zonal compartments. With perfusion time, it was apparent that the hepatic, distal compartments contributed to the majority of EA elimination.
The gross levels of hepatic GSH obtained during the experiments do not give any indication of the temporal-spatial changes in GSH concentrations throughout the acinus (Fig. 5-4C). However, the series-compartment model was used to simulate the zonal GSH concentrations during the time course of liver perfusions (Fig. 5-7B). It was observed that the rates of GSH depletion were higher in the two proximal (PP) hepatic compartments during the first 30 min of perfusion. This was consistent with the greater contribution of PP compartments in EA elimination during this period (Fig. 5-7A). Between the interval of 30 - 45 min, compartments 1 - 3 became almost completely depleted of GSH, and rates of GSH consumption began to increase in the more distal compartments. Again, this was reflective of the greater role of the PV compartments in the elimination of EA during the later time periods (Fig. 5-7A).

**5.4.3.2 BSA Perfusions.** In order for the series compartment model to adequately predict the experimental observations with BSA perfusions (Fig. 5-4), it was necessary to incorporate sinusoidal binding disequilibrium and assign the value of $V_{\text{uptake}}^{\text{max}}$ in each zonal unit a value that was at least 50 times greater than that used for BSA-free perfusions (Table 5-1). Thus, in contrast to the protein-free perfusion case, $V_{\text{uptake}}^{\text{max}}$ was not obtained through scale-up from the *in vitro* hepatocyte uptake data. Rather, the model dictated that the uptake clearance of EA during BSA perfusion would have to be ~675 ml/min for the whole organ using Eq. 5-1. Given that the $Q/PS_{\text{in}}$ ratio for this scenario is 27, the ratio suggests that membrane permeability does not rate-limit the uptake of unbound EA, an inference which is incompatible with our conclusion on EA disposition by isolated hepatocytes (Tirona *et al.*, 1999). Besides the high membrane permeability for EA, values for the “on” and “off” rate constants for each of the class A and class B binding sites must be exceedingly high (Table 5-1) in order to achieve an adequate prediction of the observed data (Fig. 5-6).

The analogous simulations on EA loss and GSH consumption showed that the shape of the exponential decline of EA remained constant throughout the experiments with BSA perfusion, and little time-dependent changes in sinusoidal concentrations occurred along the porto-venous axis (Fig. 5-8A). This suggests that the rate of EA elimination by each zonal unit remains similar for the
Figure 5-5  Fits of the series compartment model to data in absence of BSA. Hepatic output of EA and EA-SG (A) and hepatic GSH content (B) during protein-free perfusions (n=3) in relation to the predicted values based on the series compartment model with zonation of GSTs (Fig. 5-1).

(A)

(B)
Figure 5-6  Fits of the series compartment model to data in the presence of BSA. Hepatic output of EA and EA-SG (A) and hepatic GSH content (B) during BSA perfusions (n=3) in relation to the predicted values based on the series compartment model with zonation of GSTs and sinusoidal binding disequilibrium (Figs. 5-1 and 5-2).
Figure 5-7  Predicted temporal gradients in sinusoidal [EA] (A) and [GSH] (B) among zonal compartments with EA (44 μM) perfusions in the absence of BSA. Lines are obtained using the series compartment model (Fig. 5-1) with metabolic zonation and parameters in Table 5-1.
Figure 5-8  Predicted temporal gradients in sinusoidal [EA] (A) and [GSH] (B) among zonal compartments with EA (44 µM) perfusions in the presence of BSA. Lines are obtained using the series compartment model with metabolic zonation and sinusoidal binding disequilibrium (Figs. 5-1 and 5-2) and parameters in Table 5-1.
duration of single pass perfusion. Simulations on zonal GSH depletion showed that the rate of GSH depletion was only modestly greater upstream in comparison with downstream units (Fig. 5-8B). By the end of the experiment, the model simulation showed that although the PP zones were more depleted of GSH than the PV counterparts, GSH contents were maintained at -1.5 and 5 mM for the PP and PV regions, respectively, at the end of the experiment.

5.4.4 GSH Histochemistry.

Mercury orange staining of unfixed cryosections resulted in intense staining with control livers. The cellular morphology of these sections was poor and inadequate for orientation of the acini. Therefore, mercury orange staining of fixed tissues was performed. Although excellent morphology was observed for fixed tissues, the staining intensity was exceedingly low even at 10 min after immersion in the mercury orange solution. Omission of the cryopreservation step produced sections with good morphologic quality but the staining intensities remained low. To determine the reason for low mercury orange staining in fixed tissues, the stability of GSH in ice-cold 4% paraformaldehyde buffer was assessed. As shown in Fig. 5-9. GSH concentrations measured by HPLC rapidly decreased to 50% of the original value by 5 min. The loss was not associated with an equivalent formation of GSSG, indicating that GSH instability was not a result of oxidation and was most likely due to chemical reactions with paraformaldehyde.

**Figure 5-9** Stability of GSH in 4% paraformaldehyde in 100 mM potassium phosphate buffer (pH 7.4) at 4°C. GSH was determined by HPLC.
5.5 DISCUSSION

In this communication, we aimed at a final integration of events on binding-debinding, transport, GST, and GSH availability and the presence of nonenzymatic GSH conjugation on the bimolecular conjugation kinetics of EA. Given that in vitro data exist (Tirona and Pang, 1999), and much of the heterogeneity factors on transport, GST, and GSH availability are clarified (Tirona et al., 1999), the integration should have been readily attainable. However, an uncertainty surrounding protein binding and debinding remains. With the intention that this would become evident upon perturbation of protein binding, we studied the hepatic clearance of EA in the presence and absence of BSA and focused on the development of a distributed-in-space model for hepatic GSH conjugation of EA. It is envisioned that the acinar GSH conjugation rates of EA would differ among the zonal regions for these two experimental sets. In absence of BSA, PV hepatocytes would be spared from exposure to EA because of high uptake by PP cells due to high membrane permeability and efficient elimination, whereas with high plasma binding, EA entry to PP hepatocytes would be curtailed such that EA is able to reach downstream hepatocytes for GSH conjugation; the same phenomenon was observed with the highly bound anionic compound bromosulfophthalein (BSP) (Chen et al., 1984, Gumucio et al., 1984), and thyroxine (Weisiger et al., 1984) in the perfused rat liver. An additional advantage of this experimental design was that it demanded appropriate modeling of the sinusoidal BSA binding kinetics of EA, an aspect of hepatic EA elimination that we had not previously characterized.

Our present data showed that the hepatic extraction ratio of EA (44 µM) was significantly modified by protein binding. The hepatic outflow of EA was higher and E decreased from 0.99 to 0.65 when albumin was added to perfusate (Fig. 5-4A). In both experimental sets, EA elimination was entirely accounted for by GSH conjugate output and was consistent with hepatic GSH depletion rates (Fig. 5-4). A relatively high hepatic extraction ratio (E_ex = 0.65), however, prevailed despite the low unbound fraction (equilibrium value of f_u = 0.02) in 1% BSA. Given that GSH conjugation of EA in hepatocyte cytosol is extremely rapid (Tirona et al., 1999), this observation suggests that plasma protein binding directly modulates hepatic EA clearance by inhibiting hepatocyte influx.
In an attempt to explore the utility of established hepatic clearance models in the description of bimolecular GSH conjugation, we appraised the predictiveness of several hepatic clearance models for their estimations of EA extraction ratio during perfusion, in the presence and absence of BSA, based on in vitro hepatocyte uptake and equilibrium binding data. In the absence of BSA, space models such as the “parallel tube” and “dispersion” models closely predicted the observed data and were superior to the “well-stirred” model (Table 5-2). This result emphasizes the fact that the choice of hepatic clearance model can profoundly affect the estimated whole-organ clearance when scaling-up from in vitro data (Yamazaki et al., 1996) and suggests that space models are more appropriate. Similarly, others have shown the utility of the “dispersion model” for predicting hepatic uptake in vivo from in vitro hepatocyte uptake data for a number of compounds, including pravastatin (Yamazaki et al., 1996), grepafloxacin (Sasabe et al., 1997), ONO-1301 (Imawaka and Sugiyama, 1998), and octreotide (Yamada et al., 1997). By contrast, all of the traditional models grossly underestimated the observations on EA clearance with BSA. The lack of total compliance with data suggests that none of the models used (“well-stirred”, “parallel tube” or “dispersion”) could fully account for discrepancies upon scale-up of in vitro data. Rather, the assumptions on rapid sinusoidal binding equilibrium and uptake of only unbound drug, which were innate to these models, may be invalid for the description of hepatic EA clearance (see later discussion).

Given the superiority of the space models in their prediction of EA elimination and their greater physiological relevance, we have developed a distributed-in-space model based on the series compartment approach (Gray and Tam, 1986). The model (also called the tanks-in-series model) has been previously shown to be consistent with outflow dilution profiles upon the pulse injection of substrates into the liver (Gray and Tam, 1986); similar consistency with the dispersion of the outflow curve was achieved with the “dispersion” model (Roberts and Rowland, 1987) but not with the “parallel-tube” (plug-flow reactor) model. Furthermore, the series-compartment model has successfully predicted the hepatic disposition of thyroxine (Weisiger, 1986), rhodamine B (Braakman et al., 1989), lidocaine (Saville et al., 1992) and salicylamide (Tirona and Pang, 1996) under steady-state and non-steady-state conditions. Most importantly, the series-compartment approach has been examined with respect to enzyme zonation under linear conditions (Schwab and
Pang, 1999). The favorable evidence has compelled us to employ the series compartment approach to analyze the current experimental data on the GSH conjugation of EA in liver.

In building the model, the previously acquired data on zonal transport and conjugation were employed to build the acinar (spatial) parameters of the series compartment model (Fig. 5-1). When these \textit{in vitro} parameters were scaled-up to whole liver and the acinar distributions of enzymatic conjugation activities were incorporated into the model, an exceptionally close agreement with the experimental data on BSA-free perfusions was observed (Fig. 5-5). Based on the close predictions of the input-output data and the ease of analysis with the series-compartment model the sinusoidal concentration gradient from liver inlet to outlet during the time-course of liver perfusion was estimated. As shown in Fig. 5-7, little change occurs in the shape of the exponentially declining intrahepatic EA concentration gradient during steady-state ($t > 20$ min). However, the PP compartments were initially found to be responsible for the majority of GSH conjugation as a result of the immediate exposure under entry of EA due to the relatively high membrane permeability and the rapid cellular GSH conjugation rate. Upon continued perfusion, loss of steady-state ensued, and the shape of the EA concentration gradient changed, and, by the conclusion of the experiment, little EA was conjugated by the first three compartments; the PV compartments become substantially more important to mediate the GSH conjugation of EA.

These dynamic alterations in spatial sinusoidal EA concentrations are reflective of the changes in zonal compartment GSH levels (Fig. 5-7B). During the steady-state period, GSH content rapidly declined in the first 3 compartments and begins to approach the $K_m^{GSH}$ (1.2 mM, Tirona and Pang, 1999), whereas GSH in the distal compartments was depleted at slower rates. The observation is consistent with the role of upstream compartments in EA GSH conjugation buffering changes in the PV region during the early periods of perfusion. Eventually, the PP compartments became completely depleted of GSH and drug elimination in these compartments was no longer tenable. At this point, PV compartments became exposed to higher sinusoidal EA concentrations, resulting in increased GSH depletion rates as this zone was recruited to mediate GSH conjugation.

An important message came into focus from the distributed-in-space analysis. There is a change in the rate-limiting step (RLS) of GSH conjugation with time; the RLS changes from
cellular uptake to GSH availability during perfusion. Importantly, the RLS differs among hepatocytes along the sinusoidal plate as the perfusion progresses. By the end of the experiment the RLS in EA elimination is the co-substrate availability for PP hepatocytes and cellular uptake for the PV hepatocytes. These findings underscore the complex interplay of all the factors affecting EA elimination and emphasize the importance of temporal-spatial considerations in modeling GSH conjugation in liver.

Because all of the traditional hepatic clearance models are poor predictors of data with BSA perfusion, we modified the series compartment clearance model to test the idea that sinusoidal binding disequilibrium could explain the observations. With this scheme, adequate fits to the data could only be achievable under the circumstance that the $V_{\text{max}}^{\text{uptake}}$ was adjusted to values at least 50 times the scaled-up estimate. Subsequently, adjustment of $k_{\text{on}}$ and $k_{\text{off}}$ rate constants for binding at high and low affinity sites on BSA were constrained so that the model predictions would correspond with the observations (Fig. 5-6). The optimized values of $k_{\text{on}}$ and $k_{\text{off}}$ were much greater than the measured rate constants for other solutes in their interactions with BSA (Weisiger 1993; Nijssen et al., 1994). Despite the agreement of the model simulations and the experimental findings, the parameter values for membrane permeability and BSA binding are unreasonably high and suggest that sinusoidal binding disequilibrium might not be a realistic explanation for the plasma protein effects. Nonetheless, our attempt of the model to simulate the sinusoidal EA and cellular GSH concentrations gradients in presence of BSA were able to match the steady extraction ratio of EA and GSH contents (Fig. 5-6) and further demonstrate a more shallow gradient of EA loss in the sinusoid (Fig. 5-8A) and less striking difference in GSH depletion rates between PP and PV hepatocytes (Fig. 5-8B). Protein binding of EA buffered the changes of EA loss and depletion of GSH during the experiment, and levels of GSH in any of zones were not depleted that dramatically when compared to the instance when albumin was absent (Fig. 5-7B).

Based on the implausible parameter values required for the sinusoidal binding disequilibrium model, the possibility arises of a direct effect of BSA on facilitating EA uptake. Some other mechanisms which would be consistent with this facilitation explanation are (1) albumin receptor(s), (2) conformational adaptability of albumin through interactions with cell
surfaces, and (3) albumin mediated changes in membrane fluidity (Weisiger 1993). To interpret the effect of protein binding on EA clearance, one should be aware that this phenomenon is not unique to EA because this exists for other highly bound organic anions such as bilirubin, bromosulfophthalein, taurocholate, rose bengal, and indocyanine green (Weisiger, 1993). However, the general mechanism for the apparent facilitation of solute uptake by BSA is currently unknown. Until such mechanism is clarified, appropriate modeling of the influence of protein binding on hepatic clearance of EA cannot be accomplished.

One key element of our proposed model validation was the measurement of acinar GSH concentrations during the course of EA perfusions. Many attempts were carried out using mercury orange for the histochemical detection of GSH in liver biopsies (Asghar et al., 1975; Forkert and Moussa, 1989). Unfortunately, none was successful. Because this type of data could further validate the proposed kinetic model for EA, the void constitutes an important goal for future experimental efforts. However, if zonal depletion is observed for EA, it would not be the first observation on this phenomenon. Thurman and colleagues have used micro-light technology to assess the zonal GSH conjugation of chloro-dinitrobenzene (CDNB) in the perfused rat liver (Schön et al., 1988), and observed that GSH conjugation occurred mostly in the proximal hepatocytes along the direction of flow during antegrade and retrograde liver perfusions. Similarly, Gumucio and colleagues (Chen et al., 1984; Gumucio et al., 1981) have shown the GSH conjugation of BSP occurs mainly in upstream hepatocytes during prograde and retrograde liver perfusion when BSA is absent in perfusate.

In conclusion, we have shown, through our comprehensive in vitro investigation on uptake and metabolism and their zonal expression and intensive modeling of perfusion data from whole organ, that protein binding modulates the hepatic clearance of EA in the perfused rat liver by limiting hepatocyte influx. The common models of hepatic clearance that consider both rapid equilibration of protein binding within the sinusoids and transport of unbound ligand were incapable of predicting the influence of protein binding on EA elimination. However, sinusoidal binding disequilibrium effect cannot fully account for the clearance modulation by BSA. The
nature of this apparent facilitation of drug uptake by BSA must be examined in future in order to fully understand its impact on drug clearance.

For the analysis of GSH conjugation in whole organ, we have developed a series-compartment model based on scaled-up in vitro data and the zonal expression on transport, metabolism, and GSH availability. Simulations with this model indicate that a PP to PV GSH depletion pattern would occur during the time course of perfusion in absence of BSA. Moreover, the model shows that the rate-limiting step for GSH conjugation can differ among the zonal hepatocytes at different times during perfusion, oscillating from cellular uptake to co-substrate availability. Verification of the predicted zonal GSH depletion kinetics with zonal GSH levels would further validate the proposed model.

5.6 APPENDIX

5.6.1 BSA-free perfusions

The following mass balance rate equations were used in the series-compartment model (Fig. 5-1) of hepatic EA elimination.

The equations describing each i\textsuperscript{th} sinusoidal compartments for EA and EA-SG are,

\[
\frac{d[EA]_p}{dt} = (Q[EA]_p - \frac{V_{\text{uptake}}[EA]_p}{K_m + [EA]_p} - P_{\text{diff}}[EA]_p + P_{\text{diff}}[EA]_c - Q[EA]_p)/V_p \quad (5-A1)
\]

\[
\frac{d[EA-SG]_p}{dt} = (Q[EA-SG]_c CL_{EA-SG}[EA-SG]_c - Q[EA-SG]_c)/V_p \quad (5-A2)
\]

whereas the equations describing each cellular compartments are

\[
\frac{d[EA]_c}{dt} = \frac{V_{\text{uptake}}[EA]_p}{K_m + [EA]_p} + P_{\text{diff}}[EA]_p - P_{\text{diff}}[EA]_c - k_2[EA]_c[GSH]_c V_c

- \frac{V_{\text{metab}}[EA]_c[GSH]_c}{K_m E+A [GSH]_c + K_m GSH [EA]_c + [EA]_c[GSH]_c})/V_c \quad (5-A3)
\]
\[
\frac{d[EA-SG]_c}{dt} = (k_1 [EA]_c [GSH]_c V_c + \frac{V_{\text{metab}} [EA]_c [GSH]_c}{K_m^{\text{GSH}} K_m^{\text{EA}} + K_m^{\text{EA}} [GSH]_c + K_m^{\text{GSH}} [EA]_c + [EA]_c [GSH]_c}) - CL_b^{\text{EA-SG}} [EA-SG]_c \frac{[EA-SG]_c}{V_c} \quad (5-A4)
\]

\[
\frac{d[GSH]_c}{dt} = (-k_1 [EA]_c [GSH]_c V_c + \frac{V_{\text{metab}} [EA]_c [GSH]_c}{K_m^{\text{GSH}} K_m^{\text{EA}} + K_m^{\text{EA}} [GSH]_c + K_m^{\text{GSH}} [EA]_c + [EA]_c [GSH]_c})/V_c \quad (5-A5)
\]

and for the Bile Compartment the equations for EA-SG is,

\[
\frac{d[EA-SG]_b}{dt} = \left( \sum_{i=1}^{b} CL_i^{\text{EA-SG}} [EA-SG]_c - Q_{\text{bile}} [EA-SG]_p \right)/V_B \quad (5-A6)
\]

where \([EA]_c\), \([EA-SG]_c\), and \([GSH]_c\) are the concentrations of EA, EA-SG and GSH in each of the compartments (subscripts P, C, and B for sinusoidal, cellular and bile, respectively) of a particular zonal segment (superscript i). For the first sinusoid compartment (i = 1), \([EA]_c^{-1} = C_{in}\) and \([EA-SG]_c^{-1} = 0\). The time-dependent values of \(Q_{\text{bile}}\) are derived from Eq. 5-6. All other parameters are defined in Table 5-1.

5.6.2 BSA Studies

For experiments involving BSA perfusate, a sinusoidal disequilibrium binding model refinement was introduced (Fig. 5-2). The additional equations that govern the EA concentrations in plasma on high affinity (A) and low affinity (B) binding sites on BSA ([EA\_boundA] and [EA\_boundB] respectively) in the \(i^{th}\) sinusoidal compartments are as follows:

\[
\frac{d[EA\_boundA]_p}{dt} = (k_{\text{on}} [EA\_unbound]_p [BSA\_unbound]_p V_p - k_{\text{off}} [EA\_boundA]_p V_p + Q [EA\_boundA]_p [-1] - Q [EA\_boundA]_p)/V_p \quad (5-A7)
\]

\[
\frac{d[EA\_boundB]_p}{dt} = (k_{\text{on}} [EA\_unbound]_p [BSA\_unbound]_p V_p - k_{\text{off}} [EA\_boundB]_p V_p + Q [EA\_boundB]_p [-1] - Q [EA\_boundB]_p)/V_p \quad (5-A8)
\]

where \([BSA\_unbound]_p\) and \([BSA\_unbound]_p\) and the concentrations of free high and low affinity binding sites on BSA respectively while
\[ B_{\text{max}}^A = [EA_{\text{boundA}}]^i_p + [BSA_{\text{unbound}}]^i_p \] (5-A9)

and

\[ B_{\text{max}}^B = [EA_{\text{boundB}}]^i_p + [BSA_{\text{unbound}}]^i_p \] (5-A10)

The concentration of unbound EA in each \( i \)th sinusoidal compartment is given by

\[
\frac{d[EA_{\text{unbound}}]^i_p}{dt} = (k_{\text{effA}}[EA_{\text{boundA}}]^i_p V_p + k_{\text{effB}}[EA_{\text{boundB}}]^i_p V_p - k_{\text{out}}[EA_{\text{unbound}}]^i_p [BSA_{\text{unbound}}]^i_p V_p \\
- k_{\text{inB}}[EA_{\text{unbound}}]^i_p [BSA_{\text{unbound}}]^i_p V_p - \frac{V_{\text{uptake}} [EA_{\text{unbound}}]^i_p}{K_{\text{uptake}} + [EA_{\text{unbound}}]^i_p} - P_{\text{diff}} [EA_{\text{unbound}}]^i_p \\
+ P_{\text{diff}} [EA]^c + Q[EA_{\text{unbound}}]^i_p - Q[EA_{\text{unbound}}]^i_p) / V_p
\] (5-A11)

For model simulation, the following constraints were assigned

\[ K_D^A = \frac{k_{\text{effA}}}{k_{\text{out}}} \] (5-A12)

\[ K_D^B = \frac{k_{\text{effB}}}{k_{\text{out}}} \] (5-A13)

5.7 STATEMENT OF THE SIGNIFICANCE OF CHAPTER 5

In this chapter we determined that plasma protein binding affects the hepatic clearance of EA by inhibiting EA influx into hepatocytes. In addition, we established that the series compartment model provided the most comprehensive and predictive model of hepatic EA disposition that was consistent with \textit{in vitro}, zonal hepatocyte and whole organ data. Analysis with this model suggests that, with continued exposure of hepatocytes to EA, the rate limiting step in GSH conjugation transitions from uptake to co-substrate availability and that the rate limiting step can be different among hepatocytes at different times.
CHAPTER 6

DISCUSSION AND CONCLUSIONS
In vitro to in vivo scaling has been employed successfully to describe the hepatic removal of substrates undergoing oxidative metabolism (see reviews by Wilkinson, 1987; Houston and Carlile, 1997; Ito et al., 1998) and phase II reactions such as glucuronidation and sulfation (see review by Pang and Chiba, 1994), but is less common for GSH conjugation. Scale-up procedures usually require the selection of an appropriate hepatic clearance model. The traditional models of hepatic clearance, namely the “well-stirred”, “parallel tube” and “dispersion” models are several approaches that offer integration of in vitro data for scale-up. However these models do not readily take into account the complexity of non-linear transport and enzymatic reactions. In addition, the “well-stirred” model is a departure from the physiological concept that hepatic drug removal is distributed-in-space. For substrates undergoing GSH conjugation, scaling-up of in vitro data to the whole liver level presents an additional challenge since the co-factor supply must be considered. Consequently, more complex modeling approaches that incorporate the interplay of all factors are required for a comprehensive description of hepatic GSH conjugation kinetics.

In the present investigation, we have employed a wide variety of in vitro and whole organ techniques and experimental approaches to study GSH conjugation kinetics of the model compound EA. This strategy proved successful. The experimental observations, detailed within each chapter, support several conclusions.

1) Spontaneous GSH conjugation of EA occurred rapidly under physiologic conditions. However, enzyme-catalyzed conjugation by the GSTs predominated in rat liver due to high GST activity. The non-enzymatic GSH conjugation was described by second-order reaction kinetics, whereas enzymatic conjugation was saturable with respect to GSH and substrate (EA) and was described according to a rapid equilibrium, random binding sequence, kinetic scheme.

2) Uptake of EA into rat isolated hepatocytes consisted of a saturable and linear component within the concentration range examined. The transport of EA was sodium-independent
and was inhibited by various organic anions such as taurocholate and bromosulfophthalein. These findings suggest a facilitated uptake mechanism that is distinct from ntcp and oatp1 in rat hepatocytes for the transport of EA.

3) In vitro and hepatocyte data on transport and metabolism of EA, when viewed simultaneously with the use of physiological modeling, suggests that the uptake rate limits the overall GSH conjugation of EA in perfused rat liver. However, upon continued exposure to high concentrations of EA, availability of co-substrate rate-limits GSH conjugation when GSH becomes depleted.

4) The rate of uptake of EA was similar among hepatocytes of all zones within the liver acinus. A shallow and increasing portal-to-venous gradient in GST activity towards EA was found to exist in rat liver.

5) The process governing the biliary excretion of formed conjugate (EA-SG), most likely by Mrp2, was functionally homogeneous in liver.

6) The series compartment model offered an ideal approach to integrate the composite in vitro data for prediction of local and whole organ GSH conjugation rates.

The above experimental findings, highlighted also within Chapters three to five, can now be discussed in light of the hypotheses being tested. The first hypothesis states that “in vitro metabolic data on GSH conjugation, when scaled-up, reflect the overall metabolic activity for the whole liver”. Based on the in vitro GSH conjugation data detailed in Chapter 3, the scaled-up maximum enzymatic conjugation capacity \( V_{\text{max}} \) for the whole liver (320 \( \mu \text{mol/min/liver} \)) was inconsistent with our fitted estimate from kinetic analysis of the liver perfusion data (11.6 \( \mu \text{mol/min/liver} \)). A similar observation was noted in scaling the in vitro GSH conjugation data to the hepatocyte level (Chapter 4). In both instances, in vitro scaling resulted in a large overestimation of the \( V_{\text{max}} \). The reason for these discrepancies is likely due to the poor estimation of the whole organ (and hepatocyte) enzymatic conjugation capacity \( V_{\text{max}} \) since the concentrations of EA within the liver under the experimental conditions were not high enough to
saturate metabolism. Therefore, liver perfusion experiments employing much higher EA concentrations than those used in this investigation are required to further test this hypothesis.

The next hypothesis states that "in vitro transport data from isolated rat hepatocytes, when scaled-up, reflect the uptake and excretion in the whole liver". The uptake data from isolated hepatocytes, when scaled-up to the whole liver, were capable of predicting the steady-state hepatic extraction ratio of EA during liver perfusions with BSA-free media (Chapter 5); these findings support the aforementioned hypothesis on uptake. Moreover, uptake was directly shown to rate-limit GSH conjugation in isolated rat hepatocytes (Chapter 4). Similarly, estimates of efflux clearance of EA-SG from isolated hepatocyte data (Chapter 4), when scaled-up to the whole liver, were comparable to that estimated as the biliary intrinsic clearance for EA-SG or $CL_{int}^{BSA}$ (EA-SG) (Chapter 3), further supporting this hypothesis.

The final hypothesis states that "kinetic analyses of the individual processes and their zonation are necessary to predict local GSH conjugation between the inlet and outlet of the liver in a distributed-in-space fashion". When the in vitro data on uptake, metabolism and conjugate efflux as well as their zonation (Chapters 3 and 4) were integrated into a series-compartment distributed-in-space model for describing hepatic EA disposition (see Chapter 5), the model was capable of predicting the overall hepatic disposition of EA during BSA-free perfusion, although the experimental verification of the local GSH conjugation rates was not obtained successfully. Accordingly, the hypothesis, being true or not, awaits further experimentation.

Several unanswered questions have surfaced during the course of investigation and represent possible areas for future work. One question is on the identity of the transporter(s) responsible for the basolateral uptake of EA in rat hepatocytes. A growing number of transporters capable of sinusoidal uptake of organic anions have been reported in recent years with the application of molecular biological methods (Table 6-1). Based on the lack of sodium-dependence for uptake and the lack of strong inhibition by several ntcp/oatpl substrates, it may
Table 6-1. Basolateral Organic Anion Transport Systems in Rat Liver.

<table>
<thead>
<tr>
<th>Name</th>
<th>Mechanism</th>
<th>Substrates</th>
<th>Zonation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ntcp</td>
<td>Na⁺ co-transport</td>
<td>TCA, T3, T4</td>
<td>even</td>
</tr>
<tr>
<td>Oatp1</td>
<td>GSH and/or HCO₃⁻ counter transport</td>
<td>TCA, BSP, E₁S, E₂17βG, ouabain, ochratoxin A, CRC 220, enalapril, cholate, aldosterone, ADPA, LTC₄, DNP-SG, BSP-SG, DHEAS, gadoxetate, fexofenadine, SLCT, T3, T4, temocaprilat, BQ-123, BMG</td>
<td>even</td>
</tr>
<tr>
<td>Oatp2</td>
<td>?</td>
<td>fexofenadine, E3040-S, pravastatin, TCA, cholate, digoxin, ouabain, E₁S, E₂17βG, BQ-123, T3, T4, Type II organic cations</td>
<td>PV</td>
</tr>
<tr>
<td>oat2</td>
<td>?</td>
<td>salicylate, αKG, PGE₂, MTX, PAH</td>
<td>?</td>
</tr>
<tr>
<td>oat3</td>
<td>?</td>
<td>PAH, E₁S, ochratoxin A, cimetidine</td>
<td>?</td>
</tr>
<tr>
<td>rlst-1</td>
<td>?</td>
<td>TCA</td>
<td>?</td>
</tr>
<tr>
<td>mEH</td>
<td>Na⁺ co-transport</td>
<td>cholate, taurocholate</td>
<td>slight PV</td>
</tr>
<tr>
<td>oct1</td>
<td></td>
<td>NMN, monoamine neurotransmitters</td>
<td>PV</td>
</tr>
<tr>
<td>mct1</td>
<td>H⁺ co-transport</td>
<td>lactate, pyruvate, acetoacetate, β-hydroxybutyrate</td>
<td>PP</td>
</tr>
<tr>
<td>pepT1</td>
<td>H⁺ co-transport</td>
<td>ACE inhibitors, cephalosporins</td>
<td>?</td>
</tr>
</tbody>
</table>

* Ntcp, sodium-dependent taurocholate transporting polypeptide; oatp, organic anion transporting polypeptide; oat, organic anion transporter; rlst, rat liver specific transporter-1; mEH, microsomal epoxide hydrolase; oct, organic cation transporter; mct, monocarboxylate transporter; pepT1, peptide transporter 1

Substrates: TCA, T3, T4, TCA, triiodothyronine; T4, thyroxine; BSP, bromosulfophthalein; E₁S, estrone sulfate; E₂17βG, estradiol-17β-glucuronide; LTC₄, leukotriene C₄; DNP-SG, dinitrophenyl glutathione conjugate; BSP-SG, bromosulfophthalein glutathione conjugate; DHEAS, dihydroepiandrosterone-sulfate; SLCT, sulfoliathocholytaurine; BMG, bilirubin mono-glucuronide; αKG, α-ketoglutarate; PGE₂, prostaglandin E₂; MTX, methotrexate; PAH, paraminohippurate; NMN, N-methyl-nicotinamide; ACE, angiotensin converting enzyme; APDA, N-(4,4-azo-n-pentyl)-21-deoxyajmalinum.

PV, periportal; PP, perivenous

Sekine et al., 1998; Kusuhara et al., 1999; Abe et al., 1999, Kakyo et al., 1999
von Dippe et al., 1996; Fei et al., 1994
be concluded that neither ntcp or oatp1 is involved. The use of cell lines expressing other anion transporters (e.g. oatp2, oat2, rlst, mct1) and uptake studies with radiolabeled EA would prove useful to identify the transport protein(s) responsible for EA uptake by rat hepatocytes. Once those proteins are identified, it would be of interest to study the molecular mechanisms involved in the transport process, since it would govern the kinetics of transport (e.g. bidirectionality, counter-ion dependence).

Another question that has emerged is the mechanism by which BSA facilitates EA uptake into hepatocytes. This is a problem faced by many investigators studying the hepatic clearance of highly cleared, and highly protein bound, organic anions (see review by Weisiger, 1993). It is likely that a biophysical approach will be required to understand this phenomenon. If a new mechanism is found, this would provide a basis for appropriate kinetic modeling for the interactions between BSA, EA and hepatocytes and improve predictions of hepatic clearance.

The impact of the accumulation and efflux of the diastereomeric EA-SG conjugates in the liver is another question that needs to be addressed. Recent evidence showed that some GSTs preferentially form only one EA-SG diastereomer. The possibility that the conjugate efflux pump is stereospecific raises the possibility that further differences exist on how the cell handles GSH conjugates that are formed spontaneously and enzymatically. Our assay methodologies are not capable of discerning the EA-SG diastereomers, and hence our conclusion on GSH conjugate efflux may require modification. EA-SG diastereomers could serve as model compounds for the study of stereoselective transport; these results are applicable to other conjugates generated from electrophiles containing α,β-unsaturated ketone functionalities.

Finally, a question arises as to whether product inhibition by EA-SG occurs in liver upon exposure to EA. Our in vitro findings suggest the efficient efflux of EA-SG in hepatocytes and rapid biliary excretion in perfused liver. When hepatocytes are exposed to EA at high concentrations (such as 200 μM), a transient accumulation of conjugate may result, whereas at lower concentrations of EA, the EA-SG formed will not overwhelm the capacity of the hepatocyte
for excretion. In the event of product inhibition of GSTs by EA-SG, the spontaneous reaction, which could be rapid at high GSH concentrations, would allow for continued GSH conjugate formation and further exacerbate inhibition of the GSTs. However, in the absence of experimental data, we can only speculate on the role of product inhibition in affecting hepatic GSH conjugation rates. Eisai hyperbilirubinemic rats (EHBR), deficient in Mrp2, may provide an experimental model to test the impact of cellular EA-SG accumulation on hepatic GST activity.
REFERENCES


Fehske KJ, Muller WE (1986) High-affinity binding of ethacrynic acid is mediated by the two most important drug binding sites of human serum albumin. *Pharmacology* **32**: 208-213.


LIST OF PUBLICATIONS


APPENDIX i.

SEQUESTERED ENDOPLASMIC RETICULUM SPACE FOR SEQUENTIAL METABOLISM OF SALICYLAMIDE:
COUPLING OF HYDROXYLATION AND GLUCURONIDATION

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Drug Metabolism and Disposition 24: 821-833, 1996.

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SEQUESTERED ENDOPLASMIC RETICULUM SPACE FOR SEQUENTIAL METABOLISM OF SALICYLAMIDE

Coupling of Hydroxylation and Glucuronidation

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(Received November 7, 1995; accepted March 7, 1996)

ABSTRACT:

The metabolic disposition of simultaneously delivered [14C]salicylamide (SAM) (100 μM) and a tracer concentration of its hydroxylated metabolite [14C]gentisamide (GAM) was studied with single-pass perfusion followed by recirculating rat liver perfusion (10 ml/min). The use of dual radioisotopes and recirculating perfusion allowed for characterization of the differential metabolism of preformed [14C]GAM and formed [14C]GAM, which arose in situ in the liver with [14C]SAM single-pass perfusion, and the behavior of circulating [14C]GAM, which behaved as a preformed species in recirculation. In both modes of perfusion, [14C]GAM was mainly metabolically deaminated to [14C]glucuronides, whereas [14C]GAM predominantly formed [14C]GAM-5-sulfate. The steady-state and time-averaged clearances of GAM were identical and approached the value of flow, yielding a high hepatic extraction ratio (EF = 0.88). The apparent extraction ratio of formed GAM (EFm) = 0.88 was greater than that of the preformed species (EFm) = 0.7. Because the coupling of (SAM) oxidation and (GAM) glucuronidation was a plausible explanation for the observation, a new physiological pharmacokinetic model was developed to interpret the data. In this model, the liver was divided into three zonal units, within which acinar distribution of enzymatic activities was considered, namely portal perfusion, evenly distributed glucuronidation, and perivascular hydroxylation. Each zonal region was subdivided into extracellular, cytosolic, and endoplasmic reticulum compartments, with cytosolic (sarotransferases) and microsomal (cytochromes P-450 and UDP-glucuronosyltransferase) enzymes being segregated intracellularly into the cytosolic compartment and endoplasmic reticulum compartment, respectively. These simulations provided a good prediction of the present experimental data as well as previously obtained data with increasing SAM concentration and retrograde flow and supported the contention that SAM oxidation and GAM glucuronidation are coupled.

A substrate entering the liver may undergo successive metabolic reactions before the metabolites ultimately appear in bile and the venous effluent (1, 2). These reactions are said to occur in a distributed-in-space fashion, directed by flow along the sinusoidal flow path. Hepatic clearance models consistent with such spatial gradients within the liver, namely the parallel-tube and dispersion models or their variants, suggest that the extent of sequential removal (or extraction ratio) for the formed metabolite would be less than that for its performed counterpart in flow-limited systems (3, 4). This was indeed illustrated by the precursor-product pair phenacetin and acetaminophen in the single-pass perfused rat liver preparation (5). In contrast, the converse holds true when metabolite enters into the liver by diffusion limited (6). However, there are examples of sequential metabolism that do not conform to the aforementioned paradigms. These are found in the sequential metabolism of diazepam and SAM in single-pass perfused mouse/rat liver preparations (7–10). In these examples, the formed metabolites (nor-diazepam and GAM) are lipophilic/neutral compounds suspected of flow-limited distribution, but the extraction ratios of the formed metabolites exceed those for the performed metabolites.

The various factors that influence SAM and GAM metabolism have been thoroughly explored. Prograde (flow from the portal vein to the hepatic vein) and retrograde (flow from the hepatic vein to the portal vein) liver perfusion studies with SAM and GAM revealed trends consistent with the periporal enrichment of sulfation activities, evenly distributed glucuronidation systems, and a perivenous localization for SAM hydroxylation activities (11, 12). SAM was highly metabolized by parallel pathways to form its SAM-S and SAM-G conjugates and the hydroxylated metabolite GAM. GAM, when given to the liver, formed primarily GAM-SS and GAM-2S conjugates and formed GAM-5G only with increasing concentration (fig. 1). Similarly, the extent of metabolism for each pathway in SAM metabolism was a function of the input SAM concentration. At low concentrations, SAM-S was the major product; at increasingly higher input concentrations, disproportionate increases in SAM-G and GAM formation ensued (7). This phenomenon was attributed to the high-affinity, low-capacity SAM sulfation in the periporal region, the evenly distributed, low-affinity, high-capacity SAM glucuronidation, and the perivenous low-affinity, low-capacity SAM hydroxylation. Single-pass perfusion experiments further revealed that SAM was exclusively sequentially metabolized to the secondary metabolite GAM-5G, an observation that contrasted with the predominant formation of GAM-SS and GAM-2S from preformed GAM delivered to the liver.

This work was supported by the Medical Research Council (MRC) of Canada (MA-B104); R.G.T. was the recipient of a Pharmacy Alumni Graduate Studies Award, a Postgraduate Pharmacy Fellowship from Merck Frosst Canada, and the Pharmaceutical Manufacturers Association of Canada-MRC Postgraduate Fellowship.

Abbreviations used are: SAM, salicylamide; SAM-S, salicylamide-sulfate; GAM-G, gentisamide-glucuronide; GAM, gentisamide; GAM-2S, gentisamide-2-sulfate; GAM-5G, gentisamide-5-sulfate; GAM-5G, gentisamide-5-gluconide; ER, endoplasmic reticulum; ERC, endoplasmic reticulum compartment; CC, cytosolic compartment; EC, extracellular compartment; AUC, area under the curve; B/P ratio, blood/plasma partitioning ratio.

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TIRONA AND PANG

![Diagram of metabolic pathways for SAM](image)

all under comparable input conditions (7). When the data for parallel and sequential metabolism of SAM in porcine and rat liver preparations were modeled with a parallel-tube model that incorporated the acinar heterogeneity in sulfation, glucuronidation, and hydroxylation with Michaelis-Menten metabolism, predictions from this enzyme-distributed model were generally excellent for the primary metabolism of SAM (or preformed GAM) but not for the sequential metabolism of SAM to GAM metabolites (11). Specifically, the model failed to predict the observation of the exclusive sequential glucuronidation of GAM arising from SAM. Instead, GAM sulfate conjugates were predicted to result from SAM single-pass delivery. Another inconsistency of the model was its failure in predicting the almost complete extraction of formed GAM (E[1/1]), extraction ratio of formed GAM = 1), which exceeds that found for the preformed GAM (E[1/1]) (7, 11). Because diffusion-limited entry is not likely to exist for GAM, a neutral species, intracellular coupling of enzymatic reactions has been suggested as an explanation for the above observation (11). The subcellular localization of cytochromes P-450 (13) (for SAM hydroxylation) and UDP-glucuronosyltransferases (14) (for GAM glucuronidation) within the smooth ER and the proximal location of active sites may enhance the enzymatic coupling in the sequential metabolism of SAM. Moreover, GAM formed in the vicinity of the ER may be sequestered in the ER membrane, rendering metabolism more likely to occur via glucuronidation than sulfation, because phenol sulfotransferases are cytosolic in origin.

In this communication, we re-examined the metabolism of [14C]SAM (100 μM) and tracer concentrations of preformed [3H]GAM in combined single-pass and recirculating rat liver perfusions. Unlabeled SAM (100 μM) was co-perfused because, at this concentration, GAM was formed and detectable as a metabolite. Moreover, a much higher level of radioactive [14C]SAM was used to allow better identification of the [14C]GAM conjugates, including [14C]GAM sulfate conjugates. Using an experimental design similar to that of Pang and Gillette (5), we used simultaneous perfusion of [14C]SAM (bulk and radiotracer) and [3H]GAM (radiotracer) in single-pass and then recirculation modes, in sequence. This dual-radio-tracer approach allowed for detailed evaluation of the differential metabolism of formed and preformed GAM within a single rat liver preparation. The recirculating rat liver perfusion design was used to demonstrate the hypothesized differences in total formation of SAM secondary metabolites, in contrast to single-pass perfusion. An increased proportion of GAM sulfates during recirculation, compared with that from single-pass perfusion of [14C]SAM, was expected, because the formed [14C]GAM that escaped sequential metabolism would be more prone to sulfation than glucuronidation upon its return to the liver as a preformed species. To evaluate our observations, we developed a novel physiological model that incorporates subcellular division of the cytosolic and ER spaces in hepatic zonal regions (zones 1, 2, and 3). This model, when adapted to consider intracellular and zonal metabolic activities, was shown to adequately describe the experimental findings.

Materials and Methods

Materials. Unlabeled SAM was purchased from Sigma Chemical Co. (St. Louis, MO); [14C]SAM (specific activity, 0.87 mCi/mmol) was synthesized from [14C]salicylic acid (New England Nuclear, Boston, MA) according to the method of Mandel et al. (15). [14C]SAM was purified by TLC (silica gel GF, 100 μm; Analtech, Newark, DE) in a system of chloroform/methanol/ammonium hydroxide (40:20:1, v/v/v) and its radiochemical purity, determined by radioactivity elution profile in HPLC, was found to be >99%. Unlabeled GAM was synthesized as described by the method of Morris and Levy (16). [3H]GAM (specific activity, 6.13 mCi/mmol) was produced by catalytic reduction with sodium borohydride (New England Nuclear). [14C]GAM was purified by TLC (silica gel GF, 250 μm; Analtech), developed with ethyl ether. The radiochemical purity of [14C]GAM, determined by HPLC, was found to be >99%. Bovine serum albumin (25% solution in Tyrode’s buffer) was obtained from Sigma. All solvents were of HPLC grade (Fisher Scientific or Caledon Laboratories, Mississauga, Ontario, Canada), and other reagents were of analytical grade.

In Situ Rat Liver Perfusion. Nonfasted male Sprague-Dawley rats (Charles River Canada, St. Constant, QC, Canada) (220–330 g) were killed, blood was collected and the liver maintained at 37°C with a heat lamp. Surgery was performed under pentobarbital anesthesia (50 mg/kg, ip), and the surgical procedure and perfusion apparatus were identical to those described previously (7). Each rat was perfused via the portal vein and the perfusate exited via the hepatic vein. Bile was collected via a cannula inserted into the bile duct. The perfusion medium (pH 7.4) consisted of 20% washed human red blood cells (Red Cross, Toronto, Ontario), 1% bovine serum albumin, and 300 mg/dl glucose (50% dextrose injection USP, Travensol Canada, Mississauga, Ontario) in Krebs-Henseleit bicarbonate solution. The perfusate was oxygenated (95% oxygen/5% carbon dioxide) at a flow rate of 40 ml/min. The liver was consideredperfused when bile was collected at a rate of >2 ml/min, for 1 h. After perfusion, the liver was removed, weighed, and frozen in liquid nitrogen. Following thawing, the liver was homogenized in 10 mM Tris-HCl (pH 7.4) and centrifugation at 20,000 g. The supernatant was then used for the assay of metabolites and the rate of metabolism was determined by the method of Pang and Gillette (5).

Design of Experiments. Each experiment (n = 5) consisted of two phases (I and II). In phase I, the rat liver was perfused in single-pass fashion (40 min) with perfusate containing [14C]SAM (260,000 ± 30,000 dpm/ml, 106 ± 12 μM) and tracer [3H]GAM (100,000 dpm/ml) in two of these experiments. The concentration of SAM used in these studies was chosen on the principle that higher concentrations of SAM would produce detectable amounts of GAM. Preliminary experiments showed that the time required to reach steady state was <15 min. Two samples were taken from the reservoir at 0 and 15 min, and the mean of the determinations was used to determine the SAM steady-state input concentration, C0. Four consecutive samples were obtained from the hepatic venous outflow at 5-min intervals beginning at 22.5 min, which corresponded to the midpoint time for bile collection. The mean of the outflow steady-state GAM concentrations was denoted as Cs. Bile was collected at 30 min intervals, into previously weighed glass vials, at 20 min of phase I. After the completion of the single-pass experiment (40 min), phase II was initiated by returning the outflow perfusate to a second perfusate reservoir (250 ml), which contained the same concentrations of SAM (bulk and radiolabeled) and [3H]GAM as for single-pass perfusion. For phase II, 2–3 ml of perfusate were sampled from the reservoir at 0, 5, 10, 15, 30, 45, 60, 90, and 120 min.
COUPLING OF SEQUENTIAL METABOLISM

The volume removed (\(\sim 20 \text{ ml}\)) was <10% of the total reservoir volume and no attempt was made to correct for the loss in volume. Bile was collected into preweighed glass vials at 5-10 min intervals, such that the mid-time of the interval corresponded to the reservoir sampling time. The difference in weight before and after bile collection was taken to be the volume of bile, assuming that the density of bile is 1.0 g/ml. Blood perfusate was extracted within 3 hr of each experiment to avoid triethyl loss of GAM due to exchange. Samples of the plasma perfusate, obtained upon centrifugation of the blood perfusate, and bile were stored at \(-20^\circ\text{C}\) until analysis.

Quantitation of SAM and Metabolites in Perfusate Blood, Plasma, and Bile. The concentrations of SAM and GAM in blood perfusate and of SAM, GAM, and their metabolites in plasma and bile were determined by the HPLC methods described previously (17). Blood perfusate (1-2 ml) and authentic standards containing SAM (0.1-20 \(\mu\text{g}\)), GAM (0.1-5 \(\mu\text{g}\)), and varying known amounts of \([\text{U}]^3\text{H}\)SAM and \(\text{[}^3\text{H}\)GAM were brought to a common volume (2 ml) with blank perfusate, if required. After the addition of internal standard (5 \(\mu\text{g}\) of \(\text{pH}\)-isoproterenol), the samples were extracted with 8 ml of ethyl acetate, and, after drying of the organic phase under \(N_2\), the sample was reconstituted with 200 \(\mu\text{l}\) of the mobile phase before injection into the HPLC system. Detection was at 313 nm, and eluted radioactive fractions containing labeled SAM and GAM were collected, combined with scintillation fluid (Ready Protein, Beckman Canada), and analyzed by dual-channel \(\beta\)-counting (scintillation counter 5801; Beckman) with automatic quench correction by an external-standard method. A portion of the plasma sample (100 \(\mu\text{l}\)) was directly counted, and an additional 400-\(\mu\text{l}\) aliquot was precipitated with 2 ml of methanol. After centrifugation, the supernatant was removed, dried under \(N_2\), and reconstituted with mobile phase before undergoing liquid chromatography. Eluted fractions corresponding to radioactivity SAM and GAM and their metabolites were collected and subjected to liquid scintillation counting. Bile samples were diluted with 1:1 (v:v) with water, and 25-50-\(\mu\text{l}\) aliquots were directly counted. A portion of the diluted bile (25-50 \(\mu\text{l}\)) was directly injected into the HPLC system, followed by liquid scintillation counting of the radioactivity. Sample recovery was made by comparison of the HPLC-eluited count/direct count.

Background activities of <3 times the background count were treated as zero. All \(^{14}\text{C}\) and \(^{3}\text{H}\) quantified in samples were >1000 and >3000 dpm, respectively. Because the specific activity of SAM was the same for the input and output samples during single-pass perfusion and identical for all samples obtained in the reservoir during recirculation, quantitation of outflow \(\text{[}^3\text{H}\)SAM and \(\text{[}^3\text{H}\)GAM metabolites was made by virtue of the specific activity.

Calculations. The B/P ratio was considered for mass balance. All perfusion output rates were expressed in relation to blood flow and blood concentrations and then finally to the input rate (blood perfusate flow \(\times\) input blood concentration) or dose. The B/P ratios previously determined at our laboratory for SAM (1-3), GAM (3, 7), [\text{U}]\text{H}\)SAM, [\text{U}]\text{H}\)GAM, and [\text{U}]\text{H}\)GAM metabolites were made by virtue of the specific activity.

Duration phase 1 (single-pass perfusion), the biliary excretion rate for all species was determined as the concentration in bile at steady state multiplied by the bile flow rate (volume of bile collected divided by the time interval for each collection). Formation rates of the terminal metabolites (conjugates) were estimated as the summed efflux rates in bile and outflow plasma (flow \(\times\) outflow concentration \(C_{\text{ow}}\), at steady state, and these were further expressed as a percentage of the input rate of SAM or GAM. The extraction ratios for SAM (E) or performed GAM (E\text{(p)}\text{mm}) were expressed as the difference between the inflow \(C_{\text{in}}\) and outflow \(C_{\text{ow}}\) concentrations divided by the outflow concentration, at steady state. The total formation rate of the primary metabolite, GAM, was given by the sum of formation rates of GAM and its metabolites GAM-2S, GAM-3S, and GAM-5S. The apparent extraction ratio of formed GAM (E\text{(p)}\text{mm}) was calculated as the total efflux rate of GAM divided by the summed efflux rate of all GAM species. Clearance was given by the product of blood flow rate (Q) and E. The biliary excretion clearances of SAM and GAM during single-pass perfusion were given by the biliary excretion rate divided by the biliary concentration of the respective specific activity.

During phase II (recirculation), the time-averaged clearance of SAM and performed GAM was estimated by the ratio of dose divided by AUC\text{GAM\text{tmax}}, where AUC\text{GAM\text{tmax}} was determined by the linear trapezoidal rule and AUC\text{GAM\text{tmax}} was estimated by dividing the concentration at 120 min by the elimination constant obtained upon regression of the logarithmic-linear phase. The extraction ratio was calculated as the time-averaged clearance divided by Q. The cumulative amount of GAM and its metabolites excreted in bile at each reservoir sampling time was estimated by the area under the excretion rate vs. time curve, between 0 and 120 min (or 40-160 min of perfusion time), and was expressed as a percentage of the dose (260 \(\mu\text{g}\) in the recirculating period) to identify that at the end of phase I. The amount of metabolite in the reservoir was given by the product of the concentration and the volume of the reservoir. The total amount of metabolite formed at each sampling time was estimated as the sum of the cumulative amount excreted in bile and the amount remaining when the reservoir, expressed as a percentage of the dose (260 \(\mu\text{g}\) in the recirculating period) was identical to that at the end of phase I.

Modeling and Simulations. A series of liver models (models A, B, C, and D) were tested for their abilities to predict both \([\text{U}]^3\text{H}\)SAM and \(\text{[}^3\text{H}\)GAM metabolism (fig. 2). For recirculation, a reservoir compartment was added to the models (phase II of each study). Model A was a simple well-stirred model that contained an EC and an intracellular compartment wherein metabolism occurred (fig. 2A). When examined, this model was unsuitable because it predicted that E\text{(p)}\text{mm} > E\text{(mm)} and failed to describe the metabolism of \([\text{U}]^3\text{H}\)SAM and \(\text{[}^3\text{H}\)GAM. Model II is a series compartment model containing three sequential units, each containing an EC and an intracellular compartment. This model is a simplified form of the parallel-tube model for SAM previously published by Xu and Pang (11) (fig. 2B). When no zonal distribution of enzymatic activity or 260 \(\mu\text{g}\) of CPM GAM was placed in each of the three sequential units, the model predicted that 40 \(\mu\text{mol} > E\text{(p)}\text{mm} \text{[}^3\text{H}\)GAM. With inclusion of heterogeneous enzymatic activities (peripheral sulfation and even glucuronidation and perevious hydroxylation) and Michaelis-Menten metabolism in this model, model predictions are slightly modified, and the results are expected to model those obtained by Xu and Pang (11). Model C is a refinement of model A, where two cellular, expressed as a percentage of the dose (260 \(\mu\text{g}\) in the recirculating period) was identical to that at the end of phase I. The apparent biliary excretion clearance of SAM was calculated by the biliary excretion rate divided by the mid-point reservoir concentration of each respective species.
FIG. 2. Schematic representation of the pharmacokinetic models (A, B, C, and D).
The acinar distribution of enzymatic activities toward sulfation, glucuronidation, and oxidation used in model D is shown.
COUPLING OF SEQUENTIAL METABOLISM

TABLE 1

<table>
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<th>Parameter</th>
<th>Description</th>
<th>Assigned Value</th>
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<td>Verc (ml)</td>
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<td>Intercompartment transfer clearances</td>
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<td>CTlsother (ml/min)</td>
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<td>Total hepatic metabolite formation clearances</td>
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<tr>
<td>CLl[GAM-5G] (ml/min)</td>
<td>Total hepatic GAM-5G formation clearance</td>
<td>15.0*</td>
</tr>
</tbody>
</table>

* Experimental data.
† Obtained from Schwab et al. (20).
‡ Obtained from Pang et al. (21) and St. Pierre et al. (22).
§ Assigned parameter.
¶ Adapted from Pang et al. (23).
² See fig. 1 for acinar distribution of metabolite formation clearances.

The trend, however, allowed us to adopt a simplistic view of SAM metabolism; the formation rates for all metabolites, including that for SAM-S, were treated as first-order reactions. The formation clearances for sulfation, glucuronidation, and hydroxylation are thus proportionally adjusted according to the fractional metabolic clearance of SAM (fig. 2D).

The rates of formation in the perfusate outflow and bile were summed, normalized to the input rate/dose, and modeled. Because the formation of each metabolite was not preceded on its biliary excretion, modeling of biliary excretion was not performed. The procedure avoided additional assumptions on values of the biliary intrinsic clearances (CLintrinsic) for SAM and all of the metabolites.

For both single-pass and recirculation designs, mass-balanced rate equations were developed to describe the net transfer for each compartment (see Appendix). These differential equations were solved numerically using the software package SCIENTIST (version 2; MicroMath Scientific Software, Salt Lake City, UT). The parameters and values used for simulation have been optimized by trial and error and are summarized in table 1. A transmembrane clearance of 150 ml/min was assigned for SAM (21); for GAM, a lower value of 17 ml/min was used. For the assessment of uncertain parameters (for example, transmembrane clearances and transfer clearance across intracellular pools, i.e. the cytosol and ER), simulations were first performed for the single-pass (phase I) mode for constant delivery of SAM and perforemed GAM; the parameters were altered, refined, and optimized to closely match the experimental data. With the recognition that the liver contained parent drug and metabolites at the commencement of recirculation, the hepatic compartments at time 0 of recirculation were assigned values corresponding to those found at the end of the single-pass simulation.

Statistical Analysis. The paired Student t test was used to compare the kinetic and metabolic data for [14]C]SAM and [14]C]GAM and their metabolites during single-pass perfusion and recirculation. The level of significance was set at 0.05.

Results

Sequential Metabolism of [14]C]SAM. SAM was found to be highly cleared during single-pass perfusion (E = 0.98 ± 0.10), and values for E were similar to those obtained with recirculation (E = 0.97 ± 0.06, p = 0.82) (table 2). The concentrations of SAM and its metabolites in plasma for phase I and II are given in fig. 3A. During single-pass perfusion, the outflow plasma concentrations of all species reached steady state; SAM-S was the major metabolite, followed by SAM-G, GAM-5G, GAM-5S, and then GAM-2S. Within 120 min of recirculation, a monexponential decline of SAM (t1/2 = 18.1 ± 0.6 min) to 1% of its initial concentration was observed; accumulation of all metabolites to plateau values was further observed in reservoir plasma, and the ultimate accumulation of each metabolite was similar to that for single-pass perfusion. Only small amounts of unconjugated [14]C]GAM (<0.3% of the SAM input rate) were detected in the outflow perfusate during phase I, whereas in phase II GAM was detected only in the reservoir of one experiment (experiment 1; data not shown).

Biliary excretion rates for the glucuronides SAM-G and GAM-5G were generally faster than those for the sulfate conjugates and unconjugated GAM and SAM (fig. 3B). During recirculation, the cumulative excreted amounts of all species increased with perfusion time and reached asymptotic levels similar to those attained at steady state (fig. 3C). SAM and its metabolites represented only 4.6% of the SAM input rate during phase I and 6.3% of the dose during phase II (p > 0.05), with SAM-G, GAM-G, and SAM-S being major components in both phases.

Differences in the extent of formation of each primary metabolite, found by summing the rates or amounts in perfusate and bile (pmol per minute or pmol) and expressing these as fractions of input rate or dose, were not statistically different (p > 0.05) between single-pass perfusion and recirculation. When the plasma and biliary outflow rates/amounts were combined, SAM-S was found to be the predominant primary metabolite formed, followed by SAM-G and then SAM-OH or GAM (sum of GAM, GAM-2S, GAM-5S, and GAM-5G) during both study phases (table 2). Formed GAM (E[ml] = 0.96 ± 0.03) appeared to be more highly extracted than preformed GAM (E[ml] = 0.7) during
single-pass perfusion. During recirculation, the absence of \(^{14}C\)GAM in the reservoir in all but one experiment precluded the accurate estimation of the apparent extraction ratio of formalized GAM, \(E(mL)\). The extent of formation of each secondary metabolite was not different between single-pass perfusion and recirculation (\(p > 0.05\)), whereas GAM-5G was found as the major secondary metabolite, followed by GAM-5S and GAM-2S (table 2). The sulfate to glucuronide ratio (sum of GAM-2S and GAM-5S/GAM-5G) during phase I (1.0 ± 0.6) was not significantly different from that during phase II (0.5 ± 0.2) (\(p = 0.14\)).

**Metabolism of Preformed Tracer \(^{3}H\)GAM.** Because of extensive loss of the tritium label due to exchange, data for labeled tracer GAM were reliable only for experiments 1 and 2; data obtained for other preparations (experiments 3 to 5) were not reported. Due to the limited number of reliable experiments, statistical analysis of the data was not performed. In the two experiments, \(^{3}H\)GAM was moderately extracted during single-pass perfusion and recirculation (mean \(E(pmL) = 0.736\) and 0.642, respectively). During single-pass perfusion, steady state was reached, as shown by the constancy of outflow concentrations of \(^{3}H\)GAM metabolites (fig. 4A) and concentrations in bile (fig. 4C). During recirculation, \(^{3}H\)GAM declined monoeaxonentially (mean \(t_{1/2} = 26 \text{ min}\) to approximately 7% of the initial concentration at the end of 120 min of recirculation; \(^{3}H\)GAM metabolites accumulated in the reservoir perfusate (fig. 4A), as observed for \(^{14}C\)SAM. In bile, the major metabolite component was GAM-5G during single-pass perfusion and recirculation (fig. 4B); and, again, the glucuronide was excreted faster than the sulfate conjugates (GAM-5S and GAM-2S) and unconjugated GAM. The cumulative excreted amounts of \(^{3}H\)GAM metabolites during phase II increased and approached asymptotic levels similar to those attained at steady state (fig. 4C). Bilary excretion of radioactivity accounted for only 9.3% of the input rate during single-pass perfusion and 11.5% of the dose during recirculation (fig. 4C).

An assessment of metabolite formation (bile and plasma) with both perfusion modes revealed that GAM-5S was the most abundant metabolite formed, accounting for 49-52% of GAM metabolism, followed by GAM-5G (33-34%) and then GAM-2S (14-18%). No discernible trend was observed.

**Biliary Clearances of Formed Metabolites.** When the biliary excretion clearances for \(^{14}C\)SAM and its metabolites were plotted against perfusion time, little change was noted for SAM excretion during single-pass perfusion and recirculation. However, time-dependent declining excretion clearances were observed for SAM metabolites during phase II (fig. 5A). Similarly, a time-dependent decline in the biliary clearances of the \(^{14}C\)GAM metabolites was observed; again, the excretion clearances for preformed GAM were similar during both single-pass and recirculating perfusions (fig. 5B).

**Model Simulations.** After optimization of uncertain parameters, the simulations resulted in accurate predictions of the experimental data. Figs. 6 and 7 display the predicted disposition and total metabolism for \(^{14}C\)SAM and \(^{3}H\)GAM during single-pass perfusion and recirculation. The model was capable of predicting the preferential sequential metabolism of SAM to GAM-5G and the predominant production of GAM-5S from preformed GAM (table 3). In addition, the model predicted that the extraction ratio for the formalized GAM \(E(mL) = 0.92\) would be greater than that for its preformed counterpart \(E(pmL) = 0.72\) (table 3). In contrast, these trends were not well described by the enzyme-distributed model of Xu and Pang (11), for which SAM primary metabolite formation was well described; however, the model of Xu and Pang failed to predict the secondary metabolism of SAM, namely, GAM glucuronidation, and a higher \(E(pmL)\) than \(E(pmL)\) (table 3). Although not shown in fig. 6, model D predicted the presence of \(^{14}C\)GAM in the reservoir during the recirculation phase, although these concentrations were below the limit of our detection. The appropriateness of the assigned zonal gradients in volution and oxidative activities in model D was evident when previously obtained data on prograde and retrograde perfusion were examined (10, 11). The model simulations gave adequate predictions of the extraction ratios for SAM and preformed GAM in the present study as well as in those prograde and retrograde experiments previously described (7, 10-12). Furthermore, the model simulations resulted in improved estimates of the metabolite ratios for prograde and retrograde perfusion experiments involving SAM and preformed GAM (table 4).

**Discussion**

The disposition of \(^{14}C\)SAM in the present study was similar to that found in previous studies at low SAM input concentrations, where the
COUPLING OF SEQUENTIAL METABOLISM

Fig. 3. Concentration in outflow and reservoir perfusate (A), biliary excretion rate (B), and cumulative biliary excretion (C) of [14C]SAM and its metabolites during single-pass perfusion (0–40 min) and recirculation (40–160 min).

Fig. 4. Concentration in outflow and reservoir perfusate (A), biliary excretion rate (B), and cumulative biliary excretion (C) of [14H]GAM and its metabolites during single-pass perfusion (0–40 min) and recirculation (40–160 min).

Symbols, mean and SD of five experiments.

extraction ratios of SAM and formed GAM were both high and the extent of primary metabolite formation (SAM-S > SAM-G > SAM-OH or GAM) was similar to those observed previously (7, 11). However, the exclusivity in SAM sequential metabolism to form GAM-SG was not observed. Although GAM-SG remained the predominant secondary metabolite, GAM-SS and GAM-2S were also observed as secondary metabolites of SAM during a single passage through the liver. This was due to an increased detection sensitivity because larger amounts of radioactivity were used in the present experiments, compared with those used previously (~200,000 vs. ~80,000 dpm/ml).

The fractional metabolism of SAM to each primary metabolite was not different during single-pass perfusion and recirculation. The results were not expected because the input concentration of SAM used (100 μM) exceeded the Keq for sulfation (15 μM) and was comparable to those for glucuronidation and hydroxylation (151 and 281 μM, respectively) (7). Saturation of sulfation was expected to occur, especially in perportal hepatocytes. For SAM, which is highly cleared (clearance of almost 10 ml/min) and decays with a short t½ (~18 min with a reservoir size of 260 ml), desaturation of the metabolic pathways, especially sulfation, was expected for recirculation experiments. Increased SAM-S would be accompanied by reduced SAM-G and GAM formation with recirculation, compared with single-pass perfusion. Inclusion of this nonlinearity in the simulation procedure was achieved by replacement of the linear intrinsic clearance term for SAM-S formation (CLs) by Vmax/Keq + C_SAM. Indeed, the trend for increased SAM-S during recirculation was readily predicted with the nonlinear model (simulation results not shown). Because of large interanimal variability, however, the averaged data failed to show the small difference expected. Similarly, the fractional composition of secondary metabolites, or the sulfate to glucuronide ratio for GAM
metabolism, was not statistically different between phases I and II. In theory, GAM formed in situ in the liver is expected to be sequentially metabolized primarily to GAM-5S, whereas the GAM that escapes liver metabolism and re-enters the liver upon recirculation would be more prone to sulfation. However, the increased formation of GAM sulfates during recirculation, compared with single-pass perfusion, is contingent on detectable amounts of formed GAM escaping sequential metabolism and returning to the circulation and then to the liver as a preformed species. Inasmuch as the level of GAM detected in recirculating experiments was either very low or nonexistent due to the rate-sensitive apparent extraction ratio of GAM formed in situ in the liver ($E_{\text{ml}}$ = 0.96), the amount of formed GAM escaping sequential metabolism would then be too small to allow for adequate perturbation in the fractional formation of secondary metabolites during recirculation, compared with the single-pass situation.

$[^{3}H]$GAM was metabolized mainly to GAM-SS and in lesser amounts to GAM-5G and GAM-2S. These findings are consistent with previous liver perfusions of GAM in comparable input concentrations (10). The extraction ratio of approximately 0.7 for tracer GAM was much lower than that obtained previously (0.85) (10). This discrepancy could not be explained by enzyme competition with the GAM formed from SAM intracellularly, which was very low. The fractional formation of $[^{14}C]$GAM metabolites did not differ between single-pass perfusion and recirculation, suggesting linear metabolism for both $[^{3}H]$GAM and $[^{14}C]$GAM.

A time-dependent decline in the biliary excretion clearances for all SAM and GAM conjugates was observed with recirculation (figs. 5A and 5B). Because these are terminal primary and secondary metabolites formed in situ in the liver, the nascently formed metabolite that undergoes immediate excretion provides a component of clearance that is not accounted for by the concentration of the metabolite in circulation. This phenomenon was first observed for enalaprilat, formed in both liver (24) and kidney (25). A constant radial value was not reached for the metabolite excretion clearances because continued formation of metabolites occurred until the termination of the recirculation experiment.

Once again, we demonstrate that the metabolism of the exogenously delivered primary metabolite ($[^{3}H]$GAM) is different from that of the primary metabolite formed in situ, ($[^{14}C]$GAM). Preformed GAM is mainly sulfated, whereas formed GAM is predominantly glucuronidated (table 3). However, the extraction ratio of the formed metabolite was considerably greater than that for the preformed species. A similar scenario was observed for the very lipophilic N-desmethyldiazepam, the primary metabolite of diazepam, in the perfused murine liver (8, 9). The sequential metabolism of diazepam to oxazepam via N-desmethyldiazepam was greater than the metabolism of N-desmethyldiazepam to oxazepam under comparable conditions; the extraction ratio for N-desmethyldiazepam derived from diazepam (0.51) was greater than that for preformed N-desmethyldiazepam (0.4). For these examples of SAM and diazepam sequential metabolism, the experimental observations could not be explained by the well-stirred model, parallel-tube model, dispersion model, or enzyme-distributed tubular flow model (9, 11). The increased extrac-
tation ratio for formed GAM, compared with preformed GAM, could be due to diffusion-limited transport across the sinusoidal membrane, which tends to promote removal of the formed metabolite and reduce entry of the preformed species (6). Alternatively, a novel modeling approach that considers a sequestered space for enzymatic coupling may explain the observations.

Because it is well recognized that hepatic drug processing is a distributed-in-space phenomenon and that acinar localization of enzymes exists in zonal regions, the series compartment approach to hepatic clearance modeling has been extensively used and examined in the past. Weissiger and co-workers (26, 27) used this approach to describe the effects of protein binding on hepatic substrate clearance and to interpret intrahepatic sinusoidal concentration gradients. Gray and Tam (28) have shown that a series compartment model can empirically predict the elimination of a variety of substrates by the liver as well as the transit-time distribution of noneliminated reference markers during multiple-indicator dilution experiments. Braakman et al. (29) have developed a model consisting of a series of sequential units, each containing extracellular, intracellular tissue, and intracellular sequestration compartments to describe the acinar compartmentalization and plasma reapparance of the nonmetabolized substrate rhodamine B. A heterogeneous compartment model described by Saville et al. (30) was used to study mass transfer and metabolism of lidocaine and its metabolites. Finally, a physiological pharmacokinetic/pharmacodynamic model was reported by Fredenek et al. (31) to describe the disposition of ethyl acrylate. In the latter model, the liver is composed of three compartments-in-series, representing perportal, midzonal, and centrilobular regions in which ethyl acrylate could be metabolized or bound to proteins.

The model D currently under consideration incorporates many elements of the previous models and further includes the subcellular division and acinar distribution of enzymatic activity. The model could include the Michaelis-Menten metabolism that was described for SAM (7). In addition, nonlinear protein binding, previously observed with SAM (7), may also be accommodated in the model. However, because protein binding of SAM was not significant, we have disregarded the small accompanying induced change in protein binding for this highly cleared compound. The large cellular distribution space for SAM, due to saturable and nonsaturable cellular binding that created an apparent cellular distribution space of >5 times the cellular water space (23), also was not considered in the modeling. Tissue binding is not important for the single-pass phase, because tissue binding is completed at steady state and does not contribute to material loss. Moreover, a monophasic decline resulted during recirculation, as a consequence of previous loading during single-pass perfusion, and the dramatic effect of tissue binding can thus be discounted.

![COUPLING OF SEQUENTIAL METABOLISM](image)

**Fig. 7. Disposition of $^{14}$H/GAM and total formation (sum of bile and peritoneal) of its metabolites during single-pass perfusion (A) and recirculation (B).**

**Symbols:** means of two experiments; lines, derived from simulations with model D.

**TABLE 3**

Performance of model D as the prediction of GAM metabolism with prograde flow (secondary metabolism of SAM and metabolism of preformed GAM)

<table>
<thead>
<tr>
<th>Single-Pass Prograde GAM</th>
<th>Single-Pass Prograde GAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{obs}}$</td>
<td>$E_{\text{pred}}$</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Observed data</td>
<td>Predicted by enzyme-distributed model</td>
</tr>
<tr>
<td>$0.92^a$</td>
<td>$0.87^a$</td>
</tr>
<tr>
<td>$0.96^b$</td>
<td>$0.70^b$</td>
</tr>
<tr>
<td>$0.80$</td>
<td>$0.68$</td>
</tr>
<tr>
<td>$100 \mu M$ SAM</td>
<td>$0.84$</td>
</tr>
<tr>
<td>$100$ or $140 \mu M$ SAM</td>
<td>$0.72$</td>
</tr>
</tbody>
</table>

* Percentage of input rate of SAM.
* Percentage of metabolism of GAM.
* Data of Xu et al. (7), $140 \mu M$ GAM.
* Data of Morris et al. (10), $80 \mu M$ GAM.
* Present data with $100 \mu M$ SAM.
* Present data with tracer $^{14}$H/GAM.
TABLE 4

Performance of model D and enzyme-distributed model with SAM prograde and retrograde data

<table>
<thead>
<tr>
<th></th>
<th>Single-Pass Prograde SAM</th>
<th>Single-Pass Retrograde SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>SG*</td>
</tr>
<tr>
<td>Xu and co-workers (7, 11), 140 μM SAM</td>
<td>0.95</td>
<td>4.08</td>
</tr>
<tr>
<td>Present study, 100 μM SAM</td>
<td>0.98</td>
<td>4.75</td>
</tr>
<tr>
<td>Enzyme-distributed model, 140 μM to 100 μM SAM</td>
<td>0.98-0.99</td>
<td>2.92-3.70</td>
</tr>
<tr>
<td>Model D, 100 or 140 μM SAM</td>
<td>0.98</td>
<td>4.22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Single-Pass Prograde GAM</th>
<th>Single-Pass Retrograde GAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>SG</td>
</tr>
<tr>
<td>Observed data, Morris et al. (10, 12), 80 μM GAM</td>
<td>0.97</td>
<td>1.18</td>
</tr>
<tr>
<td>Enzyme-distributed model, 80 μM GAM</td>
<td>0.89</td>
<td>2.04</td>
</tr>
<tr>
<td>Model D, Tracer (10GAM)</td>
<td>0.72</td>
<td>1.21</td>
</tr>
</tbody>
</table>

* SG, sulfat; GAM, gluconate; OH, GAM.

We have, however, made several assumptions regarding the sinusoidal transmembrane (CLSm) and C/OEC transfer (CLSm) of SAM, GAM, and their metabolites (table 1). Values for the sinusoidal transfer clearances for SAM (much greater than blood flow rate) (23) and SAM-S (rapid cellular influx and efflux) (18) are reasonable because these have been well characterized in multiple-indicator dilution studies. However, similar information on transmembrane transfer of SAM-G, GAM, and GAM-SS, and GAM-5G is missing. In light of the increased hydrophilicity of the SAM metabolites, we have assigned sinusoidal transmembrane clearances for GAM and all of the conjugates to vary between that for SAM and the blood flow rate (17 ml/min). For GAM metabolites, the transmembrane clearances are not important because the formation of GAM conjugates is not predicated on these clearances and because these are terminal metabolites.

For the aforementioned conditions examined, metabolic intrinsic clearances of 6.2, 3.1, and 15 ml/min for GAM, GAM-2S, GAM-SS, and GAM-5G formation, respectively, and corresponding C/OEC transfer clearances for GAM of 8.3 ml/min (or 0.5 times the sinusoidal transfer clearance) were necessary to arrive at optimised model predictions. Again, the C/OEC clearances are not important for addressing the fate of terminal metabolites. In contrast, alteration of the C/OEC transfer clearance of GAM, and hence its C/OEC/sinusoidal transfer clearance ratio, from 0.01 to 100 without alteration of the metabolic intrinsic clearances resulted in profound effects on the sequential metabolism of SAM and the metabolism of preformed GAM (fig. 8). A slow C/OEC transfer clearance for GAM would lead to sequestration of the formed GAM arising from SAM in the C/OEC. This "entrapment" would promote further glucuronidation within that compartment. In addition, a slow C/OEC transfer clearance for SAM would depress hydroxylation and glucuronidation of SAM and preformed GAM because of slow transfer into the C/OEC. Large ER/sinusoid transfer clearance ratios would result in increased glucuronidation and hydroxylation of SAM and glucuronidation of preformed GAM. In fact, we now observe that GAM-5G becomes the major metabolite of preformed GAM, and preformed and formed GAM are metabolized in similar fashions because the intracellular space becomes well mixed. The ratio appeared to be optimal at 0.5, because these clearance values made good the model predictions on the steady state metabolic clearance of glucuronidation after SAM administration (fig. 8a) but the dominance of GAM sulfation after GAM administration (fig. 8b). In addition, this optimal ratio was consistent with the extraction ratios for formed and preformed GAM (fig. 8c). Larger C/OEC transfer clearances for GAM would reduce the enzymatic coupling between SAM oxidation and GAM hydroxylation, result in a well-mixed CC, which is not different from that proposed by Xu and Pang (11), and fail to account for the experimental findings.

One should be aware, however, that the sinusoidal transmembrane clearances, C/OEC transfer clearances, and metabolic clearances are highly interrelated, and the set of values is not unique. Needless to say, other combinations could provide equally consistent results. Additional simulations were performed to explore the role of the sinusoidal transmembrane clearance of GAM in SAM sequential metabolism. When the transfer clearance was varied from 17 to 50 ml/min (flow limitation) or 6.5 ml/min (barrier-limited), predictions similar to those obtained experimentally could be obtained. When the sinusoidal transmembrane clearance for GAM was flow-limited (50 ml/min), the associated GAM-2S, GAM-SS, and GAM-5G formation intrinsic clearances in whole liver were 2.3, 8.5, and 13.5 ml/min, respectively, and the C/OEC transfer clearance was 0.05 times the sinusoidal transmembrane clearance. When the sinusoidal clearance for GAM was barrier-limited (6.5 ml/min), the associated GAM-2S, GAM-SS, and GAM-5G formation intrinsic clearances in whole liver became 20, 75, and 195 ml/min, respectively, and the C/OEC clearance was 5 times the sinusoidal clearance. Thus, the optimal C/OEC clearance was contingent on the sinusoidal transmembrane clearance/metabolic intrinsic clearances to fit the set of observations. These added explorations showed that values for GAM permeation characteristics at the sinusoidal membrane or ER transfer need not be unique. However, the presence of the cytoplasmic and ER spaces in modelling was of paramount importance. Lack of modeling of the sequestered ER space for hydroxylation/glucuronidation would fail to fully describe the data on the metabolism of SAM and GAM.

The idea that hydroxylation and glucuronidation has coupled has been proposed in the past (10, 32). However, several other reports questioned such a supposition (33-36). For example, the phenolic substrates harmine, p-nitrophenol, and 4-hydroxybiphenyl were shown to be preferentially sulfated rather than glucuronidated when perfused through rat livers (35-37). When the metabolism of their precursor precursors harmine, p-nitroanisole, and biphenyl, respectively, were studied, the sulfated sequential product remained predominant over the glucuronidated secondary metabolite (33-36, 38). These effects were concentration-dependent; thus, enzyme kinetics can readily explain the lack of coupling observed with these substrates. In the case of SAM, studies by Xu et al. (11) revealed that, even at wide SAM input concentrations (30-1400 μM), GAM-5G remained the predominant (and only) sequential metabolite observed. Unlike the other phenolic substrates described previously, enzyme kinetics and acute heterogeneity of metabolic activities together could not account for such findings with the previous model (11). The model is robust enough to predict both the disposition of SAM and
preformed GAM and the coupling of oxidative and glucuronosidative activities toward SAM. Clearly, use of the subcellular ERC does not imply assumptions about the intracellular locations of the active sites of cytochromes P-450 (facing cytosolic segment) (13, 39) and UDP-glucuronosyltransferases (luminal segment) (40); rather, the ERC can be viewed as a compartment that may not represent an actual physiological space. Our model, which considers the ER acting as a subcellular sequestration space, may in fact be describing the partitioning of drug substrates in the ER. The creation of a subcellular ERC can be addressed by the following scenario: SAM interacts with cytochrome P-450 on the ER and is metabolized to GAM. GAM, being quite lipophilic, becomes

sequenced and concentrated in the ER membrane. Close proximity of membrane-sequestered GAM to membrane-localized UDP-glucuronosyltransferases promotes its sequential glucuronidation rather than sulfation. Direct evidence of such events that would lead to coupled metabolic reactions, however, remains to be found.

In conclusion, we showed that a primary metabolite formed in situ could be metabolized differently (in extent and subsequent metabolism), in comparison with its preformed counterpart. This behavior was due, in part, to the acinar distribution and to subcellular localization of enzymatic activities. These two factors affect the coupling of drug metabolic pathways and could explain the aberrant metabolism of formed GAM and preformed GAM derived from its precursor, SAM. Such factors must therefore be taken into account in future efforts to model the sequential hepatic metabolism of drugs.

Appendix

Glossary. The terminologies used to describe the mass-balanced differential equations for model D are as follows. \( n \) denotes the hepatic unit, where \( n = 1, 2, \text{ or } 3 \). \( C_n[X] \) denotes the concentration of species \( X \) in the reservoir, \( C_{EC}[X] \), \( C_{CC}[X] \), and \( C_{ERC}[X] \) denote the concentrations of species \( X \) in the EC, CC, and ERC, respectively, of the \( n \)th hepatic unit. \( C_{in}[X] \) represents the input concentration of species \( X \) during phase I (single-pass perfusion). \( Q \) denotes the total blood (perfusion) flow rate, \( V_{EC}, V_{CC}, \text{ and } V_{ERC} \) denote the volume of the reservoir, EC, CC, and ERC, respectively. \( CL_{EC}^X \) denotes the unidirectional membrane influx and efflux clearances for species \( X \). \( CL_{ERC}^X \) denotes the ER transfer influx and efflux clearances for species \( X \). \( CL_{CC}^X \) represents the formation clearance for the production of metabolite \( X \) in hepatic unit \( n \).

Mass-Balance Differential Equations.

Reservoir (Phase II Only).

\[
\frac{dC_{in}[X]}{dt} = \left( Q \frac{C_{EC}^X}{V_{EC}} - \frac{Q}{V_{EC}} \right) C_{in}[X]
\]

\( n \)th compartment in EC

\[
\frac{dC_{EC}[SAM]}{dt} = \left( Q \frac{C_{EC}^X}{V_{EC}} - CL_{EC}^X \right) C_{EC}[SAM] - C_{EC}[SAM] \left( Q \frac{CL_{EC}^X}{V_{EC}} \right)
\]

\[
\frac{dC_{CC}[GAM]}{dt} = \left( Q \frac{C_{CC}^X}{V_{CC}} - CL_{CC}^X \right) C_{CC}[GAM] - C_{CC}[GAM] \left( Q \frac{CL_{CC}^X}{V_{CC}} \right)
\]

\[
\frac{dC_{ERC}[SAM-S]}{dt} = \left( Q \frac{C_{ERC}^X}{V_{ERC}} - CL_{ERC}^X \right) C_{ERC}[SAM-S] - C_{ERC}[SAM-S] \left( Q \frac{CL_{ERC}^X}{V_{ERC}} \right)
\]

\[
\frac{dC_{ERC}[SAM-G]}{dt} = \left( Q \frac{C_{ERC}^X}{V_{ERC}} - CL_{ERC}^X \right) C_{ERC}[SAM-G] + \left( CL_{ERC}^X \right) C_{ERC}[SAM-G] - C_{ERC}[SAM-G] \left( Q \frac{CL_{ERC}^X}{V_{ERC}} \right)
\]

\[
\frac{dC_{ERC}[GAM-2S]}{dt} = \left( Q \frac{C_{ERC}^X}{V_{ERC}} - CL_{ERC}^X \right) C_{ERC}[GAM-2S] + \left( CL_{ERC}^X \right) C_{ERC}[GAM-2S] - C_{ERC}[GAM-2S] \left( Q \frac{CL_{ERC}^X}{V_{ERC}} \right)
\]
TIRONA AND PANG

\[
d\mathcal{C}_c^{SS}(\text{GAM-SS})/dt = [\mathcal{Q}\mathcal{C}_c^{SS}(\text{GAM-SS})] + \mathcal{C}_c^{SS}(\text{GAM-SS})\mathcal{C}_c^{SS}(\text{GAM-SS}) - \mathcal{C}_c^{SS}(\text{GAM-SS})\mathcal{C}_c^{SS}(\text{GAM-SS}) + Q)/V_{cc} \]

\[
d\mathcal{C}_c^{SG}(\text{GAM-SG})/dt = [\mathcal{Q}\mathcal{C}_c^{SG}(\text{GAM-SG})] + \mathcal{C}_c^{SG}(\text{GAM-SG})\mathcal{C}_c^{SG}(\text{GAM-SG}) - \mathcal{C}_c^{SG}(\text{GAM-SG})\mathcal{C}_c^{SG}(\text{GAM-SG}) + Q)/V_{cc} \]

For phase I (single-pass perfusion) where \( n = 1 \), \( C_{cc}^{EC} \) for SAM and GAM becomes \( C_{cc}^{EC} \), whereas \( C_{cc}^{EC} \) for the other species is given a value of zero.

For phase II (recirculation) where \( n = 1 \), \( C_{cc}^{EC} \) for all species becomes \( C_{cc}^{EC} \).

**6th compartment in CC**

\[
d\mathcal{C}_c^{6} (\text{SAM})/dt = [\mathcal{C}_c^{6}(\text{SAM})] + \mathcal{C}_c^{6}(\text{SAM})\mathcal{C}_c^{6}(\text{SAM}) - \mathcal{C}_c^{6}(\text{SAM})\mathcal{C}_c^{6}(\text{SAM}) + \mathcal{C}_c^{6}(\text{SAM})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{SAM-S})/dt = [\mathcal{C}_c^{6}(\text{SAM-S})] + \mathcal{C}_c^{6}(\text{SAM-S})\mathcal{C}_c^{6}(\text{SAM-S}) - \mathcal{C}_c^{6}(\text{SAM-S})\mathcal{C}_c^{6}(\text{SAM-S}) + \mathcal{C}_c^{6}(\text{SAM-S})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{SAM-G})/dt = [\mathcal{C}_c^{6}(\text{SAM-G})] + \mathcal{C}_c^{6}(\text{SAM-G})\mathcal{C}_c^{6}(\text{SAM-G}) - \mathcal{C}_c^{6}(\text{SAM-G})\mathcal{C}_c^{6}(\text{SAM-G}) + \mathcal{C}_c^{6}(\text{SAM-G})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM})/dt = [\mathcal{C}_c^{6}(\text{GAM})] + \mathcal{C}_c^{6}(\text{GAM})\mathcal{C}_c^{6}(\text{GAM}) - \mathcal{C}_c^{6}(\text{GAM})\mathcal{C}_c^{6}(\text{GAM}) + \mathcal{C}_c^{6}(\text{GAM})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM-SS})/dt = [\mathcal{C}_c^{6}(\text{GAM-SS})] + \mathcal{C}_c^{6}(\text{GAM-SS})\mathcal{C}_c^{6}(\text{GAM-SS}) - \mathcal{C}_c^{6}(\text{GAM-SS})\mathcal{C}_c^{6}(\text{GAM-SS}) + \mathcal{C}_c^{6}(\text{GAM-SS})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM-SG})/dt = [\mathcal{C}_c^{6}(\text{GAM-SG})] + \mathcal{C}_c^{6}(\text{GAM-SG})\mathcal{C}_c^{6}(\text{GAM-SG}) - \mathcal{C}_c^{6}(\text{GAM-SG})\mathcal{C}_c^{6}(\text{GAM-SG}) + \mathcal{C}_c^{6}(\text{GAM-SG})/V_{cc} \]

**6th compartment in ERC**

\[
d\mathcal{C}_c^{6}(\text{SAM})/dt = [\mathcal{C}_c^{6}(\text{SAM})] + \mathcal{C}_c^{6}(\text{SAM})\mathcal{C}_c^{6}(\text{SAM}) - \mathcal{C}_c^{6}(\text{SAM})\mathcal{C}_c^{6}(\text{SAM}) + \mathcal{C}_c^{6}(\text{SAM})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{SAM-S})/dt = [\mathcal{C}_c^{6}(\text{SAM-S})] + \mathcal{C}_c^{6}(\text{SAM-S})\mathcal{C}_c^{6}(\text{SAM-S}) - \mathcal{C}_c^{6}(\text{SAM-S})\mathcal{C}_c^{6}(\text{SAM-S}) + \mathcal{C}_c^{6}(\text{SAM-S})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{SAM-G})/dt = [\mathcal{C}_c^{6}(\text{SAM-G})] + \mathcal{C}_c^{6}(\text{SAM-G})\mathcal{C}_c^{6}(\text{SAM-G}) - \mathcal{C}_c^{6}(\text{SAM-G})\mathcal{C}_c^{6}(\text{SAM-G}) + \mathcal{C}_c^{6}(\text{SAM-G})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM})/dt = [\mathcal{C}_c^{6}(\text{GAM})] + \mathcal{C}_c^{6}(\text{GAM})\mathcal{C}_c^{6}(\text{GAM}) - \mathcal{C}_c^{6}(\text{GAM})\mathcal{C}_c^{6}(\text{GAM}) + \mathcal{C}_c^{6}(\text{GAM})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM-SG})/dt = [\mathcal{C}_c^{6}(\text{GAM-SG})] + \mathcal{C}_c^{6}(\text{GAM-SG})\mathcal{C}_c^{6}(\text{GAM-SG}) - \mathcal{C}_c^{6}(\text{GAM-SG})\mathcal{C}_c^{6}(\text{GAM-SG}) + \mathcal{C}_c^{6}(\text{GAM-SG})/V_{cc} \]

\[
d\mathcal{C}_c^{6}(\text{GAM-SS})/dt = [\mathcal{C}_c^{6}(\text{GAM-SS})] + \mathcal{C}_c^{6}(\text{GAM-SS})\mathcal{C}_c^{6}(\text{GAM-SS}) - \mathcal{C}_c^{6}(\text{GAM-SS})\mathcal{C}_c^{6}(\text{GAM-SS}) + \mathcal{C}_c^{6}(\text{GAM-SS})/V_{cc} \]

References


COUPLING OF SEQUENTIAL METABOLISM


APPENDIX ii.

COMPARISON OF THE DISPERSION AND GORESKY MODELS IN OUTFLOW PROFILES FROM MULTIPLE INDICATOR DILUTION RAT LIVER STUDIES

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HEPATIC CLEARANCE MODELS

Comparison of the Dispersion and Goresky Models in Outflow Profiles from Multiple Indicator Dilution Rat Liver Studies

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ABSTRACT:

The multiple indicator dilution (MID) technique is often used for investigation of the kinetic behavior of substrates and metabolites in eliminating organs. The present study was a systematic comparison of the utility of the Goresky model (GM) (a structural model) and the mixed-boundary dispersion model (DM) (a stochastic model) in the interpretation of influx, efflux, and removal (sequestration) coefficients, with data generated from rat liver-perfusion/MID studies. Although the GM and the DM are equivalent in their descriptions of membrane transport, they differ in their classification of the dispersion of blood-borne elements. For the DM, the dispersion is an inverse Gaussian distribution of vascular transit times; for the GM, it is accounted for by the dispersion observed among noneleminted reference indicators (e.g., labeled red blood cells, albumin, sucrose, and H₂O) or the derived reference. In this study, previously published rat liver-perfusion/MID data obtained for the glutathione conjugates of bromosulfophthalein and hippuric acid, compounds that exhibit saturable carrier-mediated transport, with the GM were reanalyzed with the two-compartment DM. When the fitted values for volume and transfer coefficients were compared, good correlation was found between the fitted vascular volume for the DM and the vascular volume for the reference indicator for the GM. The influx coefficients were generally similar between the models, but improved correspondence was observed when the DM was modified to include the large-vessel transit time. In contrast, the efflux and sequestration coefficients obtained for the DM did not correspond well to those from the GM. The disagreement was due, in part, to differences in the interpretation of the late-time component of the reference transit time distribution curve, which was not described well by the DM. Consequently, the residence time distribution and the relative dispersion were underestimated by the DM.

There has been major progress made in the past three decades in the field of modeling of the liver for the processing of drugs and metabolites. All of the models feature the physiological determinants of clearance, i.e., organ blood flow, vascular and tissue binding, membrane permeability, and enzymatic activity (V_max) and affinity (K_m) of intracellular enzyme systems and/or excretory apparatus for the removal of substrates. Several useful mathematical models that interrelate the deterministic variables have been developed to describe temporal events and, in particular, steady-state events, wherein drug and metabolite binding to liver tissue is completed and does not contribute to drug loss. Accordingly, the liver has been viewed as a well-mixed compartment ("well-mixed" model) (Rowland et al., 1973; Pang and Rowland, 1977) or as an organ receiving a series of nonsegregated parallel flows surrounded by identical single sheets of hepatocytes of uniform enzymatic activity ("parallel tube" model) (Winkler et al., 1973). These models have been constructed as being too extreme and idealized; because there is either infinite (well-stirred model) or no (parallel tube model) mixing, they are unable to describe the asymmetrical outflow profiles or RTDs observed after bolus dosing. The inadequacy has been explained on the basis of heterogeneities in flow (Miller et al., 1979; Bronikowski et al., 1987) and a high degree of geometric branching within the liver, which provide an intermediate mixing or dispersion. This necessitates the reselection, refinement, or development of models that could describe the observations. The barrier-limited, distributed, capillary transit time model of Goresky (Goresky, 1963; Goresky et al., 1973) and the adaptation (Roberts and Rowland, 1985, 1986) of the DM of Perl and Chinard (1968) with closed boundary conditions (Danckwerts, 1953) are such model developments. These describe curve models that resemble the outflow dilution profile observed after pulse injection, and they recognize

1 Abbreviations used are: RTD, residence time distribution; RBC, red blood cell; MID, multiple indicator dilution; GM, Goresky model; DM, dispersion model; BSP, bromosulfophthalein; HA, hippurate acid; MTT, mean transit time; CV, relative dispersion; CD, coefficient of determination; t¹, large-vessel transit time; V_b, blood volume; D_0, dispersion number; MSC, model selection criterion.
TIRONA ET AL.

The GM and the mathematical formulations have a long history, first being used for descriptions of the handling of endogenous compounds by dog liver in vivo (Goresky et al., 1973) and then being extended to analyses of xenobiotics (Schwab et al., 1990; Geng et al., 1993) and metabolic processing (Pang et al., 1994) in perfused and isolated organs involving the construction of a vascular reference curve based on an initial consideration of the binding and distribution properties of the diffusible solute in the vasculature and then appraisal of the outflow curve for the labeled tracer with respect to this reference (Schwab et al., 1990). In the past decade, the DM has been reintroduced and modified to describe substrate processing within the liver (Yano et al., 1989, 1990, 1991; Evans et al., 1991, 1995; Diaz-Garcia et al., 1992; Hussein et al., 1994; Chou et al., 1993, 1995; Yasui et al., 1993a; Ohto et al., 1996; Nishimura et al., 1996; Utaka et al., 1997). The model has been extended to the two-compartment DM, with recognition that the sinusoidal membrane of the liver is a transport barrier to solutes, producing compartmentalization of the vascular and cellular compartments.

Close examination of the GM and the DM reveals both similarities and differences; the models share common mathematical descriptions of cellular events such as transmembrane transfer, cellular drug metabolism, and biliary excretion (fig. 1), as first noted by Rowlett and Harris (1976). These models typically characterize cellular events by the use of influx (kI), efflux (kE), and sequestration (kS) coefficients (see definitions in table 1). However, the GM and the DM differ in their descriptions of the transit time distributions of their respective vascular reference curves.

Because of the pervasive use of both models in the interpretation of M1D data to provide mechanistic insight into hepatic drug processing, there is the need to fully understand the consistency of both models with the data. The intent of this report was, therefore, to compare the ramifications of the GM and the DM to gain a better understanding of the two methods and their implications for hepatic drug processing. From a practical standpoint, it is apparent that the GM is experimentally and computationally more demanding than the DM because of the requirement for noneliminated reference indicators. We therefore became interested in assessing the similarities and differences in the analysis of dilution data by the DM compared with the GM because the solutions are more readily available, with inclusion of fewer or no reference indicators. Data that had been processed by the GM with deconvolution of the sham experiments (dilution experiments without liver) were reexamined with the DM, to allow for comparison of the two models. We compared the fitted values of the common parameters (volumes and transfer coefficients) used by the GM and the DM to describe the hepatic disposition of two compounds that are bound only to albumin and that exhibited saturable sinusoidal or (carriers-mediated) transport. These compounds were BSPGSH (Geng et al., 1995) and HA, the glucose conjugate of benzene (Yoshimura et al., 1998).

Theoretical Considerations

The GM. For the GM, a set of noneliminated reference indicators ordinarily accompanies the tracer-labeled substrate for injection into the liver. The noneliminated reference indicators include 51Cr-labeled RBCs (the vascular reference, which is distributed only in the sinuoids), 125I-labeled albumin and 14C- or 3H-labeled sucrose (which occupy the sinusoidal plasma and the interstitial or Disse space), and D2O [a kinetic equivalent of water (Pang et al., 1991)] that is distributed throughout the vascular, interstitial, and accessible cellular water spaces of the organs). The outflow concentrations are normalized to the injected dose (fractional recovery per milliliter), with observations of the progressive dispersion of labeled RBCs, albumin, sucrose, and then water, based on their increasing spaces of distribution. Another

TABLE I

<table>
<thead>
<tr>
<th>Coefficient</th>
<th>Coefficients for DM</th>
<th>Coefficients for GM</th>
<th>Physical Equivalents for GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influx</td>
<td>kI</td>
<td>kI (1 + γ1)</td>
<td>V\text{plasma} \cdot \gamma_1</td>
</tr>
<tr>
<td>Efflux</td>
<td>kE</td>
<td>kE (1 + γ2)</td>
<td>V\text{plasma} \cdot \gamma_2</td>
</tr>
<tr>
<td>Sequestration</td>
<td>kS</td>
<td>kS (1 + γ3)</td>
<td>V\text{int} \cdot \gamma_3</td>
</tr>
</tbody>
</table>

* Transfer coefficients for the DM.
* Transfer coefficients for the GM and their physical equivalents.

Figure 1. Schematic representation of solute handling at the level of a single sinusoid.
well-known property of the model is the ability of the dilution curves for the diffusible noneliminated reference indicator to be superimposed on the labeled RBC curve, after appropriate correction of the time and the concentration for the difference in distribution volumes (Goreský, 1963). The procedure revealed the existence of a transit time delay ($t_0$), occurring in the nondispersing region or the large vessels, after correction for the transit times of the input and output catheters. The transit time profiles for albumin, sucrose, and water are superimposed on the RBC curve using the following relationship (Yoshimura et al., 1998).

$$h_{\text{ab}}(t) = h_{\text{bc}}(t) = \frac{1}{1 + \gamma} h_{\text{bc}} \left( \frac{1 - t_0}{1 - \gamma + t_0} \right)$$  (1)

where $h(t)$ is the transfer function that describes the RTD of the reference indicator after deconvolution of the distortion caused by the inflow and outflow catheters and the injection device. The superposition procedure, when carried out by a fitting procedure, provides the optimized fitted values of $t_0$ and $\gamma$, a value denoting the space ratios [stationary to moving spaces, i.e. Disse space/sinusoidal plasma for albumin and sucrose and (cellular water + Disse spaces)/sinusoidal plasma = RBC water] for H$_2$O; see table 1 for definition.

The basic premise of the GM for the study of propagation of tracers is the use of vascular indicators for development of an appropriate vascular reference curve. By definition, the appropriate reference curve is the hypothetical outflow profile for a given solute that would result if the solute were not taken up nor eliminated by the cells. Depending on the experimentally determined distribution of the solute between RBCs and plasma and on plasma protein binding, the reference curve is constructed from the combined behaviors of the labeled RBC, albumin, and sucrose curves, to illustrate the vascular distribution of the labeled tracer. For example, a tracer substance that is not distributed into RBCs nor bound to plasma albumin is expected to behave identically to the observed sucrose curve, in terms of the shape and the estimated vascular distribution volume. In contrast, for a drug that is highly bound to plasma protein and is not distributed into RBCs, the vascular distribution of the reference compound is similar to that for labeled albumin. By extension, a drug that is rapidly and reversibly bound to albumin but is not bound to RBCs would have a vascular reference curve that defines a distribution space intermediate between those for sucrose and albumin. In such a situation, the reference requires the construction of the following relationship (Geng et al., 1995),

$$\gamma_{\text{ref}} = f_a \gamma_{\text{ab}} + (1 - f_a) \gamma_{\text{ab}}$$  (2)

where $\gamma_{\text{ref}}$ is the apparent distribution space (interstitial/vascular space) ratio for the tracer and $f_a$ is the unbound plasma fraction, which is defined by conventional methods such as equilibrium dialysis or ultrafiltration. Another relationship is also used when the reference is described in relation to sucrose, which is used for fitting.

$$\gamma_{\text{ab}} = \frac{1 + \gamma_{\text{ab}}}{1 - \gamma_{\text{ab}}}$$  (3)

where $\gamma_{\text{ab}}$ is the factor that describes the appropriate interstitial space reference for the drug. The apparent volume for the reference curve ($V_{\text{ref}}$) is thus described with respect to that for sucrose, as

$$V_{\text{ref}} = V_{\text{ab}}(1 + \gamma_{\text{ab}})$$  (4)

where the total volume for sucrose, $V_{\text{ab}}$, can be obtained by moment analysis of the sucrose curve (catheter-corrected mean transit of sucrose $\times$ plasma flow rate).

For barrier-limited tracers, the theoretical reference transfer function may then be appropriately related to that for sucrose in describing the extracellular behavior of the labeled tracer. The organ transport function for diffusible labeled tracer can be calculated with the following equation (Yoshimura et al., 1998),

$$h_{\text{ab}}(t) = e^{-\Delta t_0} h_{\text{bc}}(t) + e^{-\Delta t_0} h_{\text{bc}}(t - \Delta t_0) \int_{t}^{t+\Delta t_0} h_{\text{bc}}(t') e^{-\Delta t_0} \, dt'$$  (5)

where $\Delta t_0$ is the delay of the cellular water space ($V_{\text{w}}$) to the sinusoidal plasma space ($V_p$) and $\theta$ is $\Delta t_0 + \gamma_{\text{ref}}$. With parameters describing the rate coefficients for influx ($J_k$, $J_k'$), efflux ($k^{-1}$), and sequestration ($k_{\text{se}}$) (see definitions in table 1). The fitting procedure furnishes estimates of $\gamma_{\text{ref}}$ and the rate coefficients. These, in turn, provide the influx and efflux rate constants ($k_i$ and $k_e$, respectively), as defined by Goreský and co-workers (Goreský et al., 1973; Geng et al., 1995; Yoshimura et al., 1998), and the permeability surface area products for influx and efflux across the hepatocyte membrane (table 1). The first term of eq. 5 represents the throughput component or material that propagates through the system, i.e. the portion of the tracer that passes through the liver without entering parenchymal cells; this throughput component is obtained from eq. 5 by setting $t = 0$. The second term represents material that enters the liver cell and later returns to the vascular compartment, or the returning component; this is given by the difference between the total outflow profile and the throughput component.

The DM. The DM conceptualizes that the blood flowing through a labyrinth of ramified interconnecting sinusoids is nonideal and causes a dispersion of noneliminated and eliminated substances (Roberts and Rowland, 1985, 1986). There are two parameters that characterize the model, i.e. $D_{\text{L}}$, or the inverse of the Pelet number, which describes the degree of mixing or dispersion resulting from heterogeneous blood flow through the microvasculature, and the efficiency number, $R_{\text{e}}$, or the term for removal.

$$D_{\text{L}} = \frac{f_{\text{CL,D}}}{Q}$$  (6)

where $f_a$ is the unbound fraction, $CL_{\text{D}}$ is the intrinsic clearance or the inherent ability for removal of the unbound substrate, and $Q$ as defined in earlier publications (Roberts and Rowland, 1985, 1986), is given by $P(P + CL_{\text{D}})$, where $P$ is the permeability. The use of $P = 1$ is justified when permeabilities far exceed the intrinsic clearance.

The dispersion observed in the outflow dilution profile for a given solute is modeled with a second-order partial differential equation that can be described in Laplace transformations after delineation of the boundary conditions. In general, the mixed-boundary DM (Roberts et al., 1988) is preferred over the closed-boundary DM of Perl and Chinard (1968) (Dancsikwits, 1953; Roberts et al., 1988) because of the ease of calculation in the former. The frequency output of a solute can be presented in the Laplace domain (Evans et al., 1991),

$$f(z) = w(z) \cdot w(z)_{\text{ref}}$$  (7)

where $z$ is the Laplace operator and $w(z)_{\text{ref}}$ and $w(z)_{\text{ref}}$ are the transfer functions that describe the spread of solute by passage through the hepatic and nonhepatic (inflow and outflow catheters, tubing, and devices) regions, respectively. The transfer function of the frequency
output, \( f(t) \) or QC(t)/dose, in the absence of the liver can be described by (Evans et al., 1991)

\[
w(s)_{\text{sham}} = \exp\left(1 - \frac{1 - \sqrt{1 + 4D_n MTT_{\text{sham}}}}{2D_n}\right)
\]

where \( D_n \) is the nonhepatic \( D_n \) and \( MTT_{\text{sham}} \) is the MTT of the reference in the sham experiment (volume of nonhepatic portion/flow or \( V_{\text{sham}}/Q \)).

The \( w(s)_{\text{sham}} \) value, or hepatic transport function of the solute, may be calculated for the one- or two-compartment DMs. The two-compartment DM is used to describe the processing of solutes that establish rapid equilibrium between the vasculature and the cells. For the noneliminated reference, which is not removed, the weighting function is

\[
w(s)_{\text{sham}} = \exp\left(1 - \frac{1 - \sqrt{1 + 4D_n V_{\text{sham}}/Q}}{2D_n}\right)
\]

where \( V_{\text{sham}} \) is the extracellular volume (combined sinusoidal and Disse spaces) and \( CL_{\text{sham}} \) is the intrinsic clearance for removal of unbound substrate.

However, with recognition that the hepatocyte membrane is a barrier that facilitates or retards the entry of solutes, producing different concentrations in the vasculature and the cells, the two-compartment model has been developed for the description (Yano et al., 1989). Elimination from the vascular (Yano et al., 1990, 1991) and/or peripheral (Yano et al., 1989; Evans et al., 1991, 1993) compartments has been considered. With the assumption that the vascular and cellular compartments are physiological spaces that are equivalent to the central and peripheral compartments, respectively, which are separated by the hepatocyte membrane, cellular removal likely occurs in the peripheral compartment. Under these circumstances, the weighting function for a solute that is distributed between the extracellular and cellular regions in liver is described as follows (Evans et al., 1991),

\[
w(s)_{\text{sham}} = \exp\left(1 - \frac{1 - \sqrt{1 + 4D_n V_{\text{sham}}/Q}}{2D_n}\right)
\]

where \( V_{\text{sham}} \) is commonly considered the extracellular volume (combined vascular and Disse spaces) and \( k_{12}, k_{21}, \) and \( k_{h} \) are the influx, efflux, and elimination coefficients, respectively (fig. 1 and table 1).

For the DM, the curve form of the vascular reference is a modified inverse Gaussian distribution, and the same function identifies the curve profile for the vascular reference. The curve form differs from that for the GM, which is constructed from the noneliminated references. For the DM, the \( V_{\text{sham}} \), the combined volume of the extracellular spaces (vascular volume + Disse space), is equivalent to the vascular volume of distribution for the reference in the GM. For the DM, the rate coefficients have been defined with respect to the compartment wherein the flux originates and have not been corrected for binding:

for the GM, all of the rate constants are defined with respect to the accessible cellular water space (\( V_{\text{cell}} \)), and the permeability surface area products have been corrected for protein binding (table 1). As in the GM, the throughput component for the DM can be obtained by setting \( k_{12} = 0 \), and the corresponding hypothetical reference curve can be determined by setting the influx parameter, \( k_{12} \), to 0 (Evans et al., 1993). Again, the difference between the total output profile and the throughput component yields the returning component. For the DM, the transit time of the noncapillary vessels (\( t_{\text{c}} \)) has not been previously considered. The absence of this delay factor in the DM might simply have been an oversight, or the factor might have been viewed as being unimportant in the distortion of the impulse. In contrast, the GM describes the large vessels as a nonexchanging and nondispersing region characterized by its transit time, \( t_{\text{c}} \).

**Materials and Methods**

Data Set. Data from single-pass, rat liver-perfusion/MID experiments were used in the present analysis. The data had been previously analyzed using the GM. In the first set of studies (\( N = 12 \)), a bolus injection of \([1\text{H}]\)BSPGSH was introduced simultaneously with the reference indicators \(^{131}\text{I}-\text{labelled RBCs,}^{125}\text{I}-\text{albumin,}^{14}\text{C}\)-glucose, and D-33) (Geng et al., 1995), under steady-state conditions, with various concentrations of BSPGSH (20–214 µM). Similarly, in another set of studies (\( N = 19 \)), \([3\text{H}]\)HA was injected, together with the set of noneliminated reference indicators, with a background concentration of HA (1–935 µM), under steady-state conditions, with or without the presence of lactate (20 mM) or benzene (10–873 µM), compounds that were found to inhibit HA uptake (Yoshimura et al., 1998). The output of \([1\text{H}]\)BSPGSH or \([3\text{H}]\)HA was expressed as the fraction of the administered dose eluting per second \( [\text{C}]/\text{dose} \) or as the frequency output \( f(t) \) or \( QC(t)/\text{dose} \).

**Data Analysis.** The frequency output was analyzed according to the DM (Evans et al., 1991). Data from sham experiments, in which labeled RBCs were injected into the inflow and outflow catheters, were used to evaluate \( w(s)_{\text{sham}} \) (eq. 8). Preliminary fits indicated that eq. 8 was unable to describe the observed exponentially multiphasic decline in the sham experiments. Therefore, \( w(s)_{\text{sham}} \) was empirically fitted to the two-compartment DM for noneliminated references where there is no elimination

\[
w(s)_{\text{sham}} = \exp\left(1 - \frac{1 - \sqrt{1 + 4D_n V_{\text{sham}}/Q}}{2D_n}\right)
\]

where \( D_n \) is the nonhepatic \( D_n \) and \( V_{\text{sham}} \) is the volume of the nonhepatic experimental system, \( Q \) is the perfusate flow rate, and \( k_{12} \) and \( k_{21} \) are the parameters accounting for the biliary decline. Least-squares fitting of the sham experiment data to eq. 12 yielded estimates of 0.0163, 1.04 ml, 0.105 sec⁻¹, and 0.388 sec⁻¹ for \( D_n \), \( V_{\text{sham}} \), and \( k_{12} \) respectively.

Because we had previously demonstrated that both BSPGSH and HA exhibit barrier limitation (carrier-mediated saturable uptake processes), the hepatic transfer function, \( w(s)_{\text{DM}} \), for the two-compartment DM (eq. 11) was used. Since only hepatic uptake and efflux occur for HA, the hepatic transport function in eq. 11 was modified by setting \( k_{12} = 0 \).

Further analysis of the outflow data was performed, in which the DM included a lag time, \( t_{\text{L}} \), that characterizes the hepatic large-vessel transit time of the GM. The \( t_{\text{L}} \) was obtained either from the GM or from the fitting procedure. In these analyses, the frequency output was described by the following equation in the Laplace domain

\[
 f(s) = w(s)_{\text{sham}} \exp(-t_{\text{L}} \cdot s) \cdot w(s)_{\text{DM}}
\]

The MTI and the CV² of the diffusible tracers were estimated for the DM according to the equations provided by Yano et al. (1989). The MTIs and variances from the GM were obtained by integration of the model functions with 1000 points using the trapezoidal rule, with or without monotonous extrapolation. The number of points was large enough that there should be no problem with accuracy. From these, correction of the MTIs and CV² for the catheter was made.
DISPERSION AND GORESKY LIVER MODELS

![Graph](image)

**Fig. 2.** Relationship between the vascular volume ($V_v$) of the DM and the volume for the reference ($V_{ref}$) of the GM for sets A, B, and C for BSGSH (data from Geng et al., 1995).

The dashed lines were established by linear regression (equations shown, with correlation coefficients $R$), whereas the solid lines are the lines of identity.

**Fitting.** The frequency outputs were fitted to eq. 7 or 13 with the use of a weighted least-squares minimization procedure, using a Levenberg-Marquardt algorithm, found in the program SCIENTIST (Micromath Scientific Software, Salt Lake City, UT). Numerical inversion of the Laplace transforms was accomplished using the Paleeva-Heymans algorithm used by the program. All parameters ($D_n$, $V_n$), the transfer coefficients, and, where appropriate, $I_0$ were estimated during the fitting procedure with the weighting choices at 1/observed value. For each experiment, the frequency output data were modeled with the two-compartment DM without inclusion of $I_0$ (set A), with $I_0$ obtained from GM (set B), and with fitted $I_0$ (set C).

**Statistical Analysis.** All data are presented as mean ± SD. The paired $t$-statistic was used for the comparison of paired data of sets A or C of the DM with those of the GM. Data of set A were compared with those of set C, because these are data fits of the DM with and without $I_0$, respectively. The MSC (SCIENTIST for Windows manual; Micromath Scientific Software) modified Akaike information criterion was used to compare the goodness of fit to the model; a larger value for MSC suggests a superior model.

**Results.**

**BSGSH Data.** Good correlation was observed between the estimated apparent $V_n$ from sets A, B, and C in the DM and the vascular volume for the reference in the GM ($R > 0.90$) (fig. 2 and table 2). However, these values (approximately 3-3.4 ml) were significantly greater than the volume for the vascular reference according to the GM (2.7-3 ml). Similarly, an excellent correlation ($R > 0.91$) was found between the influx coefficients determined by the DM ($k_{in}$) and those determined by the GM ($y_{in}$) (fig. 3). Paired analysis of the $k_{in}$ values estimated in sets A, B, and C failed to show differences between the DM and the GM ($p > 0.05$) (table 2), although clearly the correlation improved with inclusion of $I_0$ (fig. 3). In contrast, values for $k_{in}$ and $I_0$ obtained for the DM did not correspond well to the respective influx coefficients observed from the GM (figs. 4 and 5 and table 2). The DM values for $k_{in}$ and $I_0$ were higher than those from the GM (set C compared with set A, $p < 0.01$) (table 2). The addition of $I_0$ to the DM improved the correlation between the respective parameters $k_{in}$ and $I_0$ (compare fig. 4A with fig. 4, B and C, and compare fig. 5A with fig. 5, B and C) and increased $D_n$ significantly (almost 3-fold) (table 2).

The fit of the BSGSH outflow curve according to the DM was further explored by simulation of the reference curves (fig. 6A) with the fitted parameters for $D_n$ and $V_n$ for set C (see example, at a BSGSH concentration of 214 nM) and was compared with that of the GM. For the GM, the reference curve fell between that of labeled sucrose and labeled albumin, because there is substantial binding of BSGSH to albumin (unbound fraction = 0.1); the reference curve decayed slightly faster than the sucrose curve. However, for the DM, the cutofove of the reference curve decayed much more rapidly. The predicted throughput component for the DM was much smaller than that for the GM; consequently, the returning component was greater (fig. 6B). All of these discrepancies existed even though the fit of the data to the GM was only marginally better than that for the DM.

**HA Data.** Fitting was successful for all experimental data in sets A and C. However, convergence was obtained for only 14 of the 19 experiments in set B, because of the unexpectedly large $I_0$ obtained with the GM. Hence, data from only the 14 experiments were reported among all data sets. The estimates of $V_n$ for sets A, B, and C correlated well with the volume for the reference determined by the GM (3.0 ± 0.5 ml) (fig. 7 and table 2). The $k_{in}$ of the DM was moderately correlated with the influx coefficient of the GM in sets A and C (fig. 8). Inclusion of $I_0$ in the DM did not improve the correspondence for $k_{in}$ between the DM and GM estimates (compare fig. 8A with fig. 8, B and C). However, improved correspondence in the efflux parameter ($k_{out}$) was observed for sets B and C with GM values when $I_0$ was included in the DM (compare fig. 9A with fig. 9, B and C). Again, a significant increase of the $D_n$ value was observed with the inclusion of $I_0$ (table 2).

A representative fit of the indicator dilution profile for [¹³]HA (930 nM) is shown in fig. 10. For the GM, the reference curve closely followed that for sucrose (fig. 10A), owing to the modest unbound fraction of HA in plasma (unbound fraction = 0.54). The reference curve for the DM lay close to the sucrose curve at early time points and then decayed in a monoexponential fashion. The model fit to the [¹³]HA data was similar for the GM and the DM, although the GM tended to describe the late-in-time data better (fig. 10B). For [¹³]HA, the DM estimated a greater throughput and thus a smaller returning component, compared with the GM.

The MTT for BSGSH estimated with the DM was significantly smaller than that from the GM; however, similar values were found for HA (table 3). Values for CV² were generally lower for the DM than for the GM; however, significance was found only for HA and not BSGSH.

**Discussion.**

In this report, we compared the fitted values for the common parameters that characterize the hepatic disposition of two solutes.
### TABLE 2
Summary of fitted parameters from the DM (set A), DM without \( t_w \) set B, DM with GM \( t_w \) set C, DM with fitted \( t_w \) and the GM

<table>
<thead>
<tr>
<th>Volume</th>
<th>( D_w )</th>
<th>( V_w )</th>
<th>( k_{12} )</th>
<th>( k_{13} )</th>
<th>( k_{2} )</th>
<th>( CD )</th>
<th>( MSC )</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSPGSH (13 ( \pm 2 ) g liver)</td>
<td>NA</td>
<td>2.7 ( \pm 0.8 )</td>
<td>21 ( \pm 6 )</td>
<td>4.1 ( \pm 1.1 )</td>
<td>0.08 ( \pm 0.05 )</td>
<td>0.005 ( \pm 0.004 )</td>
<td>0.029 ( \pm 0.012 )</td>
</tr>
<tr>
<td>GM</td>
<td>0.04 ( \pm 0.01 )</td>
<td>3.0 ( \pm 0.6 )</td>
<td>23 ( \pm 7 )</td>
<td>0</td>
<td>0.08 ( \pm 0.03 )</td>
<td>0.013 ( \pm 0.007 )</td>
<td>0.035 ( \pm 0.009 )</td>
</tr>
<tr>
<td>DM set A</td>
<td>0.19 ( \pm 0.09 )</td>
<td>3.0 ( \pm 0.59 )</td>
<td>23 ( \pm 7 )</td>
<td>0.08 ( \pm 0.03 )</td>
<td>0.013 ( \pm 0.008 )</td>
<td>0.041 ( \pm 0.010 )</td>
<td>0.99 ( \pm 0.01 )</td>
</tr>
<tr>
<td>DM set C</td>
<td>0.15 ( \pm 0.08 )</td>
<td>3.0 ( \pm 0.59 )</td>
<td>23 ( \pm 7 )</td>
<td>3.6 ( \pm 1.2 )</td>
<td>0.08 ( \pm 0.04 )</td>
<td>0.013 ( \pm 0.007 )</td>
<td>0.041 ( \pm 0.008 )</td>
</tr>
<tr>
<td>HA (11 ( \pm 1.3 ) g liver)</td>
<td>NA</td>
<td>3.0 ( \pm 0.5 )</td>
<td>27 ( \pm 4 )</td>
<td>4.3 ( \pm 1.1 )</td>
<td>0.063 ( \pm 0.023 )</td>
<td>0.091 ( \pm 0.026 )</td>
<td>0</td>
</tr>
<tr>
<td>GM</td>
<td>0.08 ( \pm 0.05 )</td>
<td>2.9 ( \pm 0.5 )</td>
<td>26 ( \pm 3 )</td>
<td>0</td>
<td>0.069 ( \pm 0.034 )</td>
<td>0.075 ( \pm 0.016 )</td>
<td>0</td>
</tr>
<tr>
<td>DM set B</td>
<td>0.43 ( \pm 0.16 )</td>
<td>3.0 ( \pm 0.5 )</td>
<td>26 ( \pm 3 )</td>
<td>4.3 ( \pm 1.1 )</td>
<td>0.069 ( \pm 0.034 )</td>
<td>0.107 ( \pm 0.048 )</td>
<td>0</td>
</tr>
<tr>
<td>DM set C</td>
<td>0.18 ( \pm 0.09 )</td>
<td>3.0 ( \pm 0.5 )</td>
<td>27 ( \pm 4 )</td>
<td>2.2 ( \pm 1.2 )</td>
<td>0.056 ( \pm 0.015 )</td>
<td>0.073 ( \pm 0.025 )</td>
<td>0</td>
</tr>
</tbody>
</table>

\(*\) NA, not applicable.
\(^{\circ}\) Significantly different from data from the GM (\( p < 0.05 \), paired \( t \)-test).
\(^{\bullet}\) Set B significantly different from set A (\( p < 0.05 \), paired \( t \)-test).
\(^{\ddagger}\) Set C significantly different from set A (\( p < 0.05 \), paired \( t \)-test).

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**Fig. 3.** Relationship between influx coefficients \( k_{13} \) determined by the DM and the GM with the RSPGSH and HA volumes of distribution in the GM and the DM. From the various parameter estimates of the two models, several conclusions can be drawn. The first regards the similarities between the volume for the appropriate reference in the GM and the apparent volume of distribution in the DM and among the influx constants; the second, the greater than predicted dispersion of the vascular reference curves; and the third, the influence of the large-vessel transit time delay on \( D_w \) and the rate coefficients.

---

**Fig. 4.** Relationship between efflux coefficients \( k_2 \) determined by the DM and the GM for RSPGSH (data from Gring et al., 1995).
(table 2). The hypothesis that the large nonexchangeable vessels would not add to the dispersion of solutes in the liver thus appears valid.

Further support for the use of $t_0$ is provided by the excellent superposition of the curves for the diffusible noneliminated indicators onto the labeled RBC curve (scaling the time and concentration terms by the ratio of the distribution volumes) when the elapsed time is corrected for $t_0$ (Goresky, 1963). Audis et al. (1994, 1995) have demonstrated that, in the lung, virtually all of the dispersion occurs in the capillary beds and only an undetectable amount of dispersion exists in the arterial and venous trees. Taking these findings together, we conclude that the addition of $t_0$ for fitting is an appropriate refinement of the DM for hepatic modeling. However, there appears to be no consistent relationship for the $i_0$ values between the DM and the GM (fig. 11); the $i_0$ values for BSPGSH were quite similar for the DM and the GM, but the fitted $i_0$ values for the DM were consistently lower than those obtained by the superposition procedure of Goresky.

Because the large-vessel transit time could not explain the major differences in the estimates for the transfer coefficients obtained with the GM and the DM, differences in the vascular reference curves for BSPGSH or HA might be the reason for the disparities. For the DM, the reference curve is an inverse Gaussian distribution of transit times and is described by a rapid upstroke followed by a monoeponential decay; for the GM, the reference curve is molded by the shapes of curves for the appropriate reference indicators, i.e. albumin and sucrose and their dispersions within the liver, because of the partial binding of the solute to albumin (figs. 6 and 10). It was readily apparent that the reference curves for the DM and the GM were similar at early time points but diverged after 2 or 3 MTTs. The small difference in the early reference curves reflected the similarities in estimates for $k_{12}$ determined by the two models. Whereas the reference curve for the DM declined monoexponentially, the corresponding profile for the GM decayed in a fashion that closely resembled that of sucrose, with a slightly protracted tail. This difference in the downslopes of the reference curves brought about uncertainties in the throughput and returning components. There was lack of a definitive pattern observed for BSPGSH and HA with regard to the throughput and returning components (figs. 6 and 10). The throughput component is highly correlated with the magnitude of $k_{12}$ and the shape and

---

**Fig. 7.** Relationship between $V_{p}$ of the DM and $V_{p}(1 - \tau_{w})$ of the GM for sets A, B, and C for HA (data from Yoshimura et al., 1996).

The dashed lines were established by linear regression (equations shown, with correlation coefficients, R), whereas the solid lines are the lines of identity.
GM $[V_{wm}(1 + \gamma_m)]$. The closeness of the estimates suggests that the fitted value derived from the DM provides a sound estimate of the apparent volume of distribution for the solute in the vasculature (defined as "the reference" in the GM). This volume is not the combined volumes of the sinusoidal blood and the Disse space, as originally conceived. However, the difference is expected to be small, because the apparent volume of distribution for the solute approximates the total albumin space or the total sucrose space for drugs that are totally bound or unbound to albumin, respectively. Moreover, the $a$ priori assignment of $V_{wm}$ as 15% of the wet liver mass, as adopted in many reports (Evans et al., 1991, 1993), underestimates the volume for the reference in the DM and leads to poor fits (data not shown).

The influx coefficient ($k_{12}$) of the DM was found to be highly correlated with that $(f_d \cdot k_1')$ of the GM (figs. 3 and 8). The observation was the result of the similarities of the respective reference curves at the early times (upswings portions of figs. 6 and 10), such that the predictions of the two models coalesced, as noted by Rowland and Harris (1976). However, the respective reference curves eventually deviated, mostly for the later-in-time segments or the "tails" of the dilution profiles, such that the efflux and sequestration coefficients that characterize the returning component for the labeled tracer were also dissimilar. For this reason, the fit to the GM is superior (compare CD values in table 2) to that to the DM, although the fit to the DM is generally quite good, especially for the early data.

To investigate whether the large-vessel transit time could account for these differences, we included the lag time parameter $t_0$ in the DM, either as an assigned parameter (value of $t_0$ from the GM, set B) or as a fitted parameter (set C). Inclusion of $t_0$ in the DM (sets B and C) slightly improved the correspondence of influx, efflux, and sequestration coefficients between the DM and the GM. The most significant change occurred with $D_m$, which increased approximately 2–3-fold as a direct result of the incorporation of $t_0$ (table 2). This was also found in an analysis of the DM with closed- or mixed-boundary conditions, with or without $t_0$ as a fitted parameter, in rat liver-perfusion/MID studies conducted with catheters of varying lengths, in which different $D_m$ estimates were observed for the noneliminated reference indicators in the absence of $t_0$ (Schwab et al., 1998). The inclusion of $t_0$ in the DM provided a common $D_m$ (~0.22) for the noneliminated indicators (labeled RBCs, albumin, sucrose, and water), as expected with the dispersive capability of the liver. The universality of this value provides full justification for including the time delay. The finding suggests that, after the initial delay, the same extent of dispersion exists for the noneliminated references, with a common $D_m$ (varying from 0.16 to 0.31 among preparations), regardless of the distribution volumes or catheter sizes (Schwab et al., 1998). Interestingly, the goodness of fit was also improved when $t_0$ was considered in the DM in the present analysis (higher values for the CD and MSC).
dispersion and Goresky liver models

![Graphs showing dispersion models](image)

**Fig. 9.** Relationship between efflux coefficient \( (k_{12}) \) determined by the DM and the GM for HA (data from Yoshimura et al., 1998).

The dashed lines were established by linear regression (equations shown, with correlation coefficients, \( r \)), whereas the solid lines are the lines of identity.

 downslope of the reference curve. When the estimated \( k_{12} \) for the DM is greater than that for GM, the throughput component for the DM is greater than that for the GM and, conversely, the returning component is lower. A greater disparity exists for the returning component, which is obtained as a difference curve, especially when it is small. This is manifested as highly variable estimates for \( k_{12} \) and \( k_{21} \) with the DM and the GM.

It must be reemphasized that, for the GM, the reference curve is constructed from the full complement of noneliminated reference indicators, and its basis should be firmly established. Comparison of the outflow profile for the diffusible labeled tracer with that for the reference would, in essence, correct for the heterogeneities in capillary transit time and account for micromixing or geometric tortuosity, as claimed by some investigators of liver physiology (King et al., 1996; Weiss, 1997). The suitability of the model is apparent when the late-in-time data are well fitted and displayed in semilogarithmic plots (high CD values) (table 2). This was seldom performed with the DM for noneliminated reference indicators and the eliminated tracer solute. The late-in-time observations that highlight the dispersion within the system showed a systematic deviation from the fitted curve for the DM. For this reason, the MTT and \( CV^2 \) for the DM are underestimated (table 3).

However, in some instances, the tailing profile for tracers such as enalaprilat (Schwab et al., 1990) and BSPGSH in Eisa hyperbilirubinemic mutant rats (Geng et al., 1998) could not be explained by the present DM and GM but could be modeled by the presence of a deep intracellular pool (equivalent to three-compartment DM fitting; fits not shown). In other instances, the influence of tight intracellular binding or slow diffusion (Luxon and Weissger, 1993; Yasui et al.,

**TABLE 3**

<table>
<thead>
<tr>
<th></th>
<th>MTT (s)</th>
<th>CV (^2)</th>
<th>MTT (s)</th>
<th>CV (^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSGSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTT</td>
<td>5.8 ( \pm ) 4.3</td>
<td>1.00 ( \pm ) 0.00</td>
<td>25.9 ( \pm ) 3.1</td>
<td>8.7 ( \pm ) 3.1</td>
</tr>
<tr>
<td>( p^* )</td>
<td>&lt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MTT</td>
<td>17.4 ( \pm ) 3.4</td>
<td>1.10 ( \pm ) 0.72</td>
<td>26.1 ( \pm ) 3.0</td>
<td>1.09 ( \pm ) 0.35</td>
</tr>
<tr>
<td>( p^* )</td>
<td></td>
<td></td>
<td>&gt;0.05</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

* MTT corrected for inflow and outflow catheters and large-vein transit times.
\(^*\) Relative variance.
\(^*\) Two-compartment DM with fixed \( l_2 \) (set C).
\(^*\) Paired t test.
Fig. 11. Plots of the fitted \( t_v \) for the DM vs. the \( t_v \) for the GM, obtained with the supersposition procedure, for BSFGSH (A) and HA (B).

1995b) could explain the unexpected RTDs of these solutes. For the DM, it should also be recognized that parameter identification was poor for efflux and removal, especially when the presence of a deep intracellular pool was evoked (fits not shown). Moreover, one must recognize the strong interrelationships between \( V_b \) and \( k_{12} \), in addition to \( V_b \) and \( D_{in} \), with the fitting procedure.

The comparison, however, reveals the utility of the DM. To restate a few points, DM is simpler with respect to computation and experimental strategy than the GM. The volume for the reference and the influx coefficient (and therefore uptake clearance) are well estimated, and this should provide insight into the uptake mechanism for solutes. Also, the exact amount of the dose can be verified independently using the area of the reference curve, thus circumventing experimental errors in its volumetric assessment. For optimization of the DM, however, model fitting with \( t_v \) is necessary to provide improved estimates of \( V_b \) and \( k_{12} \). Furthermore, proper application of tracer methodology necessitates the use of tracers in the injection dose under steady-state conditions for the bulk unlabeled compound. The appropriateness of the curve function for the sham experiment (without liver) must be demonstrated, because it is of paramount importance in the deconvolution procedure for the tracer outflow profiles. If the simple transfer function with monoexponential decay (eq. 8) was used instead of the biphasic decay curve model (eq. 12) in the analysis of the sham experiment data for the DM in the present study, \( D_{in} \), \( V_b \), and \( k_{12} \) would be overestimated, whereas \( k_{12} \), \( k_{21} \), and \( k_{2} \) would be underestimated in the labeled tracer data (comparison not shown). Experimentally, it is necessary that data collection be extended beyond 3 MTTs, and it is imperative that the radioisotopes quantified are identical to the quantities of the named drug or its metabolites. These recommendations ensure the proper use of indicator dilution profiles in providing mechanistic insight into drug uptake processes.

References


DISPERSION AND GORESKY LIVER MODELS


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