METABOLISM OF NITROSOUREAS AND FORMAMIDES:
A PROBE INTO THEIR TOXICITY AS PRECURSORS OF REACTIVE ISOCYANATES

By

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
Division of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
December 1994

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ABSTRACT

The focus of this research was to investigate the biotransformation of nitrosoureas and formamides to isocyanates as a mechanism by which these compounds could elicit their toxicity. The metabolism of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) was studied in rats and in patients on chemotherapy; the biotransformation of N-formyl amphetamine (NFA) was examined in rats and in rat hepatic subcellular fractions; and carbamoyl thiol conjugates were investigated with respect to their reactivity and toxicity toward mitochondrial enzymatic processes.

CCNU is hydroxylated in vivo to 4-hydroxy and 3-hydroxy CCNU which, along with the parent compound, decompose to the corresponding isocyanates, namely, 4-hydroxycyclohexyl, 3-hydroxycyclohexyl, and cyclohexylisocyanate. Evidence for the formation of these reactive electrophiles in vivo was inferred from the LC/MS identification of their glutathione (GSH) and N-acetylcysteine (NAC) conjugates which were excreted in the bile and urine, respectively, of dosed rats, and as NAC conjugates in the urine of patients on chemotherapy. This GSH-dependent pathway of metabolism contributed substantially to metabolism of CCNU in rats, accounting for 14.3 ± 2.9 % of the dose of CCNU excreted in urine as carbamoylated NAC conjugates in 24 h.

BCNU decomposes in vivo to 2-chloroethyl isocyanate (CEIC) which conjugates with GSH. In support of this contention, GSH and NAC conjugates of CEIC were identified as metabolites in the bile and urine, respectively, of BCNU-dosed rats. Quantitative analysis of the urine of five patients on BCNU therapy revealed that concentrations of the NAC conjugate of CEIC varied from 5.0 to 13.6 nmol/mL.

Bioactivation of NFA to 1-methyl-2-phenylethyl isocyanate (MPIC) was investigated in rats by screening bile and urine for conjugates downstream of the phase I event. NFA was administered to rats as a mixture of protio and pentadeuteriophenyl labelled analogues to characterize the carbamoylating activity of MPIC by LC/MS contour formatting. This LC/MS
technique facilitated the identification of metabolites by presenting chromatographic and mass spectral data together as a two-dimensional array. Glutathione, cysteinylglycine, cysteine and NAC conjugates of the isocyanate MPIC were identified as biliary metabolites, whereas only the NAC conjugate was excreted in urine. The excretion of all metabolites of the mercapturate pathway in bile is a novel finding for formamides.

The catalytic activity of rat hepatic microsomes and mitochondria in the conversion of NFA to MPIC was investigated by performing incubations in the presence of GSH to trap MPIC in the form of \( S-[(1\text{-methyl-2-phenylethyl})\text{carbamoyl}]\text{glutathione} \) (SMPG), which was detected by LC/MS/MS. NFA was converted by microsomes to SMPG in a manner suggestive of cytochrome P450 catalysis. SMPG formation was marginally elevated (≤25 %) in microsomes from rats treated with acetone and phenobarbital, inducers of P450 2E1 and 2B1/2B2, respectively. In addition, microsomal conversion of NFA to SMPG was marginally affected by diethylthiocarbamate (DEDTC) and orphenadrine, mechanism-based inhibitors of P450 2E1 and 2B1/2B2, respectively. Taken together, these data suggest that neither P450 2E1 nor 2B1/2B2 play a major role in the metabolism of NFA to MPIC.

Intact mitochondria also performed the biotransformation of NFA to SMPG. We sought to demonstrate the involvement of mitochondrial P450 in this process by using sonicated mitoplasts supplemented with NADPH. Although SMPG was detected as a mitoplast product of NFA, low but significant levels of microsomal contamination did not allow the involvement of mitochondrial P450 to be unequivocally proven.

The GSH and NAC conjugates of CEIC were examined with respect to their stability, reactivity and inhibitory properties toward mitochondrial enzyme activities. \( S-[(2\text{-chloroethyl})\text{carbamoyl}]\text{glutathione} \) (SCEG) exhibited a half-life of 5.0 h in solution. In the presence of NAC, the SCEG concentration declined more rapidly (\( t_{1/2} 44 \text{ min} \)) by reaction with the free thiol to form \( N\text{-acetyl-S-[(2-chloroethyl)carbamoyl]cysteine} \) (NCEC). In the converse situation, NCEC appeared more stable than the GSH conjugate, exhibiting a half-life of 3.7 h when it reacted with GSH to form SCEG. When NCEC was administered to rats,
SCEG was identified as a biliary metabolite by LC/MS/MS. This result was considered reflective of an exchange of CEIC between NCEC and endogenous GSH.

Mitochondrial glutathione reductase (GR) was inhibited by SCEG to approximately 10% of control within 7 min, with the activity remaining depressed at 6.5 h. S-(methylcarbamoyl)glutathione (SMG), the GSH conjugate of methyl isocyanate, impaired the performance of mitochondrial respiration. State III oxidative phosphorylation, apparent in control mitochondria with ADP/O ratios of 2.4 to 3.2 (glutamate/malate as substrate) and respiratory control ratios of 3.0 to 7.2, was absent in mitochondria exposed to SMG. Taken together, these results provide evidence for the toxicity of conjugated thiol metabolites.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................... ii
TABLE OF CONTENTS ......................................................................................... v
LIST OF FIGURES .................................................................................................. vii
LIST OF SCHEMES ................................................................................................. xi
LIST OF TABLES ..................................................................................................... xiii
LIST OF ABBREVIATIONS ...................................................................................... xiv
DEDICATION ........................................................................................................... xviii
ACKNOWLEDGEMENTS ......................................................................................... xix

## I. INTRODUCTION ............................................................................................... 1

1. The Chemistry of Isocyanates ........................................................................... 2
2. Nitrosoureas ........................................................................................................ 4
3. Formamides ........................................................................................................ 11
4. Cytochrome P450 ............................................................................................... 15
5. The role of glutathione in redox homeostasis and phase II metabolism .......... 23
6. Atmospheric pressure ionization liquid chromatography/mass spectrometry (API LC/MS) .......................................................... 29
7. Thesis objectives ................................................................................................ 34

## II. EXPERIMENTAL ............................................................................................ 36

1. Chemicals and materials ..................................................................................... 36
2. Animals, surgical equipment and cell fractionation equipment ....................... 40
3. Instrumentation .................................................................................................... 42
4. Methods in chemical synthesis .......................................................................... 48
5. Biological experiments ......................................................................................... 61
6. Statistical analysis ............................................................................................... 81

## III. RESULTS ....................................................................................................... 82

1. Synthetic Chemistry ........................................................................................... 82
2. Analysis of thiocarbamate conjugates ............................................................... 87
3. Metabolism of nitrosoureas and nitrosourea-derived isocyanates ....................... 101
4. Metabolism of N-formyl amphetamine to carbamoylated conjugates in rats .. 121
5. *In vitro* metabolism of N-formylamphetamine by microsomes, intact mitochondria and mitoplasts .......................................................... 132
6. Evaluation of the reactivity and toxicity of thiocarbamate conjugates ............... 149

## IV. DISCUSSION .................................................................................................. 157

1. Analysis of GSH and NAC conjugates ............................................................... 157
2. Metabolism of nitrosoureas ............................................................................... 159
3. Metabolism of N-formyl amphetamine in rats ................................................. 169
4. *In vitro* metabolism of N-formyl amphetamine by hepatic subcellular fractions ........................................................................................................ 176
5. Toxicological significance of the reactivity of thiocarbamate conjugates... 189

V. SUMMARY AND CONCLUSIONS ................................................................. 196

VI. REFERENCES ....................................................................................... 199

VII. APPENDIX ......................................................................................... 223
LIST OF FIGURES

Figure 1. Resonance forms contributing to the isocyanate function ...........................................3
Figure 2. Structures of formamide compounds discussed in the thesis ...........................................11
Figure 3. Structures of reversible (metyrapone) and mechanism-based (orphenadrine and DEDTC) inhibitors of cytochrome P450 .....................................................20
Figure 4. Structures of compounds which undergo GSH conjugation ...........................................26
Figure 5. Schematic representation of the API LC/MS interface showing the generation of gas phase ions at atmospheric pressure and their transit to the high vacuum mass analyzer region .................................................................30
Figure 6. Diagrammatic representation of the electrospray ionization process ..............................32
Figure 7. HPLC analysis of (hydroxycyclohexyl)carbamoyl derivatives of NAC .............................84
Figure 8. API LC/MS fragment ion spectrum of S-[(4-hydroxycyclohexyl)carbamoyl]-glutathione (SFHG) obtained by CAD of MH+ at m/z 449 illustrating the fragmentation system used in this report .................................................................93
Figure 9. API LC/MS fragment ion spectra of (A) N-[(1-methyl-2-phenylethyl)-carbamoyl]-glutathione (NMPG) and (B) S-[(1-methyl-2-phenylethyl)-carbamoyl]glutathione (SMPG) obtained by CAD of MH+ at m/z 469 ................................................94
Figure 10. API LC/MS fragment ion spectrum of N-acetyl-S-[(4-hydroxycyclohexyl)-carbamoyl]cysteine (NFHC) obtained upon CAD of MH+ at m/z 305 .........................................95
Figure 11. Thermospray LC/MS of glutathione with MH+ at m/z 308 ...........................................96
Figure 12. Thermospray LC/MS of N-acetyl-S-[(4-hydroxycyclohexyl)carbamoyl]-cysteine (NFHC) with MH+ at m/z 305 .................................................................96
Figure 13. 1H-NMR spectrum of a carbamoylated NAC conjugate, typified in the case of N-acetyl-S-[(2-chloroethylcarbamoyl)]cysteine .................................................................98
Figure 14. COSY NMR spectrum of S-(cyclohexylcarbamoyl)glutathione (SCCG) .......................99
Figure 15. 13C-1H-HETCOR NMR spectrum of S-[(3-hydroxycyclohexyl)-carbamoyl]-glutathione (STHG) ........................................................................................................100
Figure 16. APCI MS of the putative metabolite SFHG, with MH+ at m/z 449, in the bile of CCNU-dosed rats at HPLC fraction tR 11.9 min .................................................................103
Figure 17. APCI fragment ion spectra of (A) synthetic SFHG and (B) the putative metabolite SFHG in the bile of CCNU-dosed rats at HPLC fraction tR 11.9 min, and present with MH⁺ at m/z 449................................. 104

Figure 18. APCI fragment ion spectra of (A) synthetic SCCG and (B) the putative metabolite SCCG in the bile of CCNU-dosed rats at HPLC fraction tR 26.5 min, and present with MH⁺ at m/z 433.................................................. 105

Figure 19. APCI MS of the putative metabolites NCCC and NFHC/NTHC, with MH⁺ at m/z 289 and 305, respectively, in the urine of CCNU-dosed rats.................. 106

Figure 20. APCI fragment ion spectra of (A) synthetic NCCC and (B) the putative metabolite NCCC in the urine of CCNU-dosed rats with MH⁺ at m/z 289.............. 107

Figure 21. APCI fragment ion spectra of (A) synthetic NFHC and (B) the putative metabolite NFHC in the urine of CCNU-dosed rats at HPLC fraction tR 8.2 min, and present with MH⁺ at m/z 305.................................................. 108

Figure 22. LC/MS SRM for the transition MH⁺/RGS for the CCNU metabolites NFHC, NTHC and NCCC in the urine of (A) a dosed rat, and (B) a patient on chemotherapy.................................................. 111

Figure 23. Typical LC/MS SRM for the transition MH⁺/RGS for a standard mixture of NFHC, NTHC and NCCC. NCEC is the internal standard.... 112

Figure 24. APCI MS of the putative metabolite SCEG, with MH⁺ at m/z 413, in the bile of BCNU-dosed rats at HPLC fraction tR 6.8 min. SCEG was also present in CEIC-dosed rats.............................. 115

Figure 25. APCI fragment ion spectra of (A) synthetic SCEG and (B) the putative metabolite SCEG in the bile of BCNU and CEIC-dosed rats at HPLC fraction tR 6.8 min, and present with MH⁺ at m/z 413.................................................. 116

Figure 26. APCI MS of the putative metabolite NCEC, with MH⁺ at m/z 269, in the urine of BCNU-dosed rats at HPLC fraction tR 9.4 min. NCEC was also present in CEIC-dosed rats.................................................. 117

Figure 27. APCI fragment ion spectra of (A) synthetic NCEC and (B) the putative metabolite NCEC in the urine of BCNU- and CEIC-dosed rats at HPLC fraction tR 9.4 min, and present with MH⁺ at m/z 269.................................................. 118

Figure 28. LC/MS SRM screen for the urinary metabolite NCEC, [³⁵Cl]MH⁺ at m/z 269, from a patient on BCNU chemotherapy.................................................. 119

Figure 29. Typical LC/MS SRM quantitation of NCEC using the transition MH⁺/RGSY₁. NCCC is the internal standard.................. 120
Figure 30. Partial LC/MS Q<sub>1</sub> contour of the bile of rats dosed with [²H₀]NFA:[²H₅]NFA. ................................................................. 124

Figure 31. LC/MS fragment ion spectrum of the biliary metabolites (A) [²H₀]SMPG, MH<sup>+</sup> at m/z 469; and (B) [²H₅]SMPG, MH<sup>+</sup> at m/z 474. ................................................................. 125

Figure 32. LC/MS fragment ion spectrum of the biliary metabolites (A) [²H₀]SMPC, MH<sup>+</sup> at m/z 283; and (B) [²H₅]SMPC, MH<sup>+</sup> at m/z 288. ................................................................. 126

Figure 33. LC/MS SRM for the biliary metabolites (A) [²H₀]SMPCG, MH<sup>+</sup> at m/z 340; and (B) [²H₅]SMPCG, MH<sup>+</sup> at m/z 345. ................................................................. 127

Figure 34. LC/MS SRM for the biliary metabolites (A) [²H₀]NMPC, MH<sup>+</sup> at m/z 325; and (B) [²H₅]NMPC, MH<sup>+</sup> at m/z 330. ................................................................. 128

Figure 35. (A) LC/MS SRM chromatograms of the biliary metabolites (a) SMPC, (b) NMPC, (c) SMPCG and (d) SMPC compared to (B) the corresponding synthetic reference compounds. ................................................................. 129

Figure 36. LC/MS SRM screening of urine for the metabolites (a) SMPC, MH<sup>+</sup>/R<sub>G</sub>S at m/z 283/122; (b) NMPC, MH<sup>+</sup>/R<sub>G</sub>SY<sub>_1</sub> at m/z 325/122; (c) SMPCG, MH<sup>+</sup>/R<sub>G</sub>S at m/z 340/179; and (d) SMPG, MH<sup>+</sup>/R<sub>G</sub>SY<sub>_2</sub> at m/z 469/179. ................................................................. 130

Figure 37. LC/MS SRM of a mixture of authentic standards of (A) SMPG and (B) NMPG. ................................................................. 131

Figure 38. Typical SRM analysis for the transitions MH<sup>+</sup>/Y<sub>2</sub> for a standard solution of (A) SMPG spiked with (B) [²H₅]SMPG. ................................................................. 138

Figure 39. Time course for the formation of SMPG as a product of hepatic microsomes prepared from untreated, phenobarbital and acetone treated rats. ................................................................. 139

Figure 40. Effects of phenobarbital (PB) and acetone treatment on rat hepatic microsomal activities: (A) SMPG formation (NFA formyl desaturation) compared to (B) p-nitrophenyl hydroxylase and (C) pentoxyresorufin O-dealkylase. ................................................................. 140

Figure 41. Effect of DEDTC on the PROD activity of microsomes prepared from acetone and PB treated rats. ................................................................. 141

Figure 42. GC/MS analysis of a standard mixture of amphetamine, [²H₅]SMPG and phentermine, extracted under alkaline conditions and derivatized as described in Experimental/Section 5.2.3.1. ................................................................. 142

Figure 43. Typical oxygraph trace showing the performance of oxidative phosphorylation by intact mitochondria. ................................................................. 144
Figure 44. LC/MS SRM screening for SMPG as a mitochondrial metabolite of NFA. 
(A) Complete system; (B) incubation without mitochondria; (C) complete system spiked with [2H5]SMPG; (D) incubation without NFA. .................................145

Figure 45. Western blot analysis of mitoplast and microsomal fractions isolated from the livers of untreated, PB-treated and acetone-treated rats.................................148

Figure 46. First order decomposition curve of SCEG in phosphate buffer (pH 7.4) at 37 °C..............................................................151

Figure 47. (A) Decomposition of SCEG (1 mM) in the presence of phosphate buffered NAC (10 mM, pH 7.4) at 37 °C with the concomitant formation of NCEC. (B) Decomposition of NCEC (1 mM) in the presence of phosphate buffered GSH (10 mM, pH 7.4) at 37 °C with the concomitant formation of SCEG..............................................................152

Figure 48. LC/MS SRM chromatograms of (A) the putative biliary components (a) SCEG and (b) NCEC, compared to (B) authentic standards of these compounds..............................................................153

Figure 49. Time course for the inhibition of mitochondrial glutathione reductase by SCEG..............................................................155

Figure 50. Oxygraph trace showing the effect of SMG (3.1 mM) on mitochondrial respiration using glutamate/malate as substrate. ..............................................................156

Figure 51. Metabolism of CCNU (50 mg/kg) in rats to carbamoylated NAC conjugates in urine over a 24 h period..............................................................164

Figure 52. Structures of compounds which undergo P450-mediated desaturation.............186

Figure 53. Comparison of the structures of SCEG and BCNU to account for the superior stability of SCEG..............................................................191
LIST OF SCHEMES

Scheme 1. Mechanism for the nucleophilic addition to isocyanates ....................... 4

Scheme 2. Chemical decomposition of nitrosoureas ............................................. 5

Scheme 3. Summary of the metabolism of BCNU and CCNU .................................. 9

Scheme 4. Metabolic pathways for the formamides DMF (Kestell et al., 1985; Gescher, 1993) and N-methyl-N-(1-methyl-3,3-diphenylpropyl)formamide (Slatter et al., 1989; Mutlib et al., 1990) ......................................................... 14

Scheme 5. Overall pathway for P450-mediated catalysis .................................. 17

Scheme 6. The glutathione redox cycle ................................................................. 25

Scheme 7. Mechanisms of GSH-mediated xenobiotic toxicity: (A) episulfonium ion formation; (B) cysteine conjugate β-lyase bioactivation; and (C) equilibrium formation of a thiocarbamate ............................................ 27

Scheme 8. Principle of citrate synthase assay showing the enzyme catalyzed conversion of acetyl CoA to CoA coupled to the cleavage of DTNB to afford the thiolate which absorbs at 412 nm .................................................. 73

Scheme 9. Hydroxylation of p-nitrophenol to 4-nitrocatechol which is detected by the absorption of light at 546 nm ................................................................. 76

Scheme 10. Monitoring of PROD activity by the formation of the fluorescent product resorufin .......................................................... 77

Scheme 11. Synthesis of thiocarbamate conjugates, typified in the case of SCCG, by nucleophilic addition of the GSH thiolate to cyclohexyl isocyanate .................... 83

Scheme 12. Synthesis of thiocarbamate conjugates, typified in the case of SFHG, by nucleophilic substitution of the 4-nitrophenoxide anion by the thiolate of GSH ......................................................................................... 83

Scheme 13. Synthesis of 3-(hydroxycyclohexyl)carbamoyl derivatives of GSH and NAC ........................................................................ 84

Scheme 14. Synthesis of (1-methyl-2-phenylethyl)carbamoyl derivatives of GSH, cysteinylglycine, cysteine and NAC ................................................................. 85

Scheme 15. Synthesis of $[^{2}\text{H}_5]$NFA and $[^{2}\text{H}_5]$SMPG from amphetamine ........... 86
Scheme 16. Synthesis of \( N\)-(1-methyl-2-phenylethylcarbamoyl)glutathione (NMPG) .................................................. 87

Scheme 17. Microsomal metabolism of NFA to MPIC which is trapped by GSH in the form of SMPG for analysis by LC/MS/MS .............................................. 134

Scheme 18. Proposed formation of carbamoylated thiol conjugates as metabolites of CCNU \textit{in vivo} ................................................................. 165

Scheme 19. Proposed pathway for the metabolism of N-formyl amphetamine in rats to carbamoylated GSH, cysteinylglycine, cysteine and NAC conjugates .............. 175

Scheme 20. Proposed mechanism for the P450-mediated oxidation of NFA ....................... 186

Scheme 21. Toxicological implications for the equilibrium formation of SCEG from CEIC and GSH ................................................................. 189
LIST OF TABLES

Table 1. Profiles of cytochrome P450 hydroxylation of CCNU in rat hepatic microsomes. ................................................................. 10
Table 2. Inducible hepatic microsomal cytochromes P450................................................................. 18
Table 3. Characteristic Q_{1} fragment ions of GSH conjugates under APCI LC/MS................. 90
Table 4. Characteristic fragment ions of carbamoylated GSH conjugates under API LC/MS/MS................................................................................. 91
Table 5. Characteristic fragment ions of carbamoylated NAC conjugates under API LC/MS/MS................................................................................. 92
Table 6. Biotransformation of CCNU in rats and in humans to the urinary metabolites NFHC, NTHC and NCCC. ................................................................. 110
Table 7. Metabolism of BCNU to NCEC in patients on BCNU/cisplatin chemotherapy. ......................... 114
Table 8. Total cytochrome P450 content of rat hepatic microsomes and mitoplasts, spectrally quantitated at the absorbance of the CO difference spectrum of the dithionite reduced enzyme................................................................. 133
Table 9. Metabolism of NFA to SMPG by microsomes prepared from untreated rats. ........ 135
Table 10. Evaluation of microsomal contamination in rat hepatic mitoplasts based on the marker enzyme KCN-insensitive NADPH-cytochrome c reductase and immunodetectable P450 2B2 and 2C11................................................................. 149
Table 11. SMPG formation by mitoplasts prepared from untreated, PB-treated and acetone-treated rats. ................................................................. 149
LIST OF ABBREVIATIONS

AMCC  
N-acetyl-S-(methylcarbamoyl)cysteine

APCI  
atmospheric pressure chemical ionization

API  
atmospheric pressure ionization

BCNU  
1,3-bis(2-chloroethyl)-1-nitrosourea

bs  
broad singlet

BSA  
bovine serum albumin

°C  
degrees Celsius

da  
circa (approximately)

CAD  
collisionally activated dissociation

CCNU  
1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea

CEIC  
2-chloroethyl isocyanate

D  
deuterium

d  
deuterium (nomenclature), doublet (NMR)

dd  
doublet of doublets

DEDTC  
diethyldithiocarbamate

DMF  
N,N-dimethylformamide

DMN  
N,N-dimethylnitrosamine

DMSO  
dimethyl sulfoxide

DTNB  
5,5'-dithiobis-(2-nitrobenzoic acid)

EDTA  
ethylenediaminetetraacetic acid

EI  
electron impact

2-ene VPA  
2-propyl-2-pentenoic acid

et al.  
et alia

EtOH  
ethanol

g  
gram (mass), gravitational acceleration, 9.8 m/s² (centrifugal acceleration)
\begin{itemize}
\item $\gamma$-GT $\gamma$-glutamyltranspeptidase
\item GC gas chromatography
\item GC/MS gas chromatography/mass spectrometry
\item GP glutathione peroxidase
\item GR glutathione reductase
\item GSH reduced glutathione
\item GSSG oxidized glutathione
\item GST glutathione S-transferase
\item h hours
\item HEPES \textit{N}-[2-hydroxyethyl]piperizine-\textit{N}'-[2-ethanesulfonic acid]
\item HFBA heptafluorobutyric acid anhydride
\item HMQC heteronuclear multiple quantum correlation
\item HPLC high pressure liquid chromatography
\item Hz Hertz
\item i.p. intraperitoneal(ly)
\item i.v. intravenous(ly)
\item J coupling constant in Hz
\item kg kilogram
\item LC liquid chromatography
\item LC/MS liquid chromatography/mass spectrometry
\item LC/MS/MS liquid chromatography/tandem mass spectrometry
\item m multiplet
\item M(H)$^+$ (pseudo)molecular ion
\item 3-MC 3-methylcholanthrene
\item mg milligram
\item MHz mega Hertz
\item MIC methyl isocyanate
\end{itemize}
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>MPIC</td>
<td>1-methyl-2-phenylethyl isocyanate</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry or mass spectrum</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetyl-L-cysteine</td>
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<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced)</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NAT</td>
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</tr>
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<td>NCCC</td>
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<td>NCEC</td>
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<td>NFA</td>
<td>N-formylamphetamine</td>
</tr>
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<td>NFHC</td>
<td>N-acetyl-S-[(4-hydroxycyclohexyl)carbamoyl]cysteine</td>
</tr>
<tr>
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<td>NMPG</td>
<td>N-[(1-methyl-2-phenylethyl)carbamoyl]glutathione</td>
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<td>NTHC</td>
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<td>pentoxyresorufin O-dealkylase</td>
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<tr>
<td>SCCG</td>
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<td>S-[(1-methyl-2-phenylethyl) carbamoyl] cysteine</td>
</tr>
<tr>
<td>SMPCG</td>
<td>S-[(1-methyl-2-phenylethyl) carbamoyl] cysteinylglycine</td>
</tr>
<tr>
<td>SMPG</td>
<td>S-[(1-methyl-2-phenylethyl) carbamoyl] glutathione</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
</tr>
<tr>
<td>STHG</td>
<td>S-[(3-hydroxycyclohexyl) carbamoyl] glutathione</td>
</tr>
<tr>
<td>t</td>
<td>triplet</td>
</tr>
<tr>
<td>t-BOC</td>
<td>tertiary butyloxy carbonyl</td>
</tr>
<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TMS</td>
<td>tetramethylsilane (NMR)</td>
</tr>
<tr>
<td>tR</td>
<td>retention time</td>
</tr>
<tr>
<td>v</td>
<td>volume</td>
</tr>
<tr>
<td>VPA</td>
<td>valproic acid</td>
</tr>
</tbody>
</table>
DEDICATION

To my family and friends...
For your constant support.
ACKNOWLEDGEMENTS

I would like to express sincere thanks to my supervisor, Dr. Frank Abbott for his support and guidance throughout this project. I am gratefully indebted to Dr. Ruedi Aebersold for access to the SCIEX API III LC/MS facility, Biomedical Research Centre, U.B.C. I am also thankful to my supervisory committee members, Dr. Raymond Andersen, Dr. Stelvio Bandiera, Dr. Keith McErlane and Dr. John Sinclair. I would like to acknowledge my colleague Wei Tang for his technical assistance and generous exchange of ideas. Special thanks to Dr. Stelvio Bandiera for the use of his laboratory; to Dr. Richard Barton, Department of Biochemistry, U.B.C., for assistance in mitochondrial respiration experiments; to Roland Burton for his assistance in mass spectrometry; to Drs. Meg Knowling (British Columbia Cancer Control Agency, Vancouver, B.C.) and Glenn Lesser and Michael Colvin (Johns Hopkins Oncology Centre, Bethesda, MD) for their clinical input; to Violet Yuen for assistance in animal handling; and to Jim "Kirk" Dibacy, Mike "Jean-Luc" Lane and Chis "Scottie" Weiz for their excellent support services. I would also like to acknowledge the various contributions of my coworkers Sashi Gopaul, Janice Moshenko, Jan Palatý, Sue Panesar and Ali Tabatabei. Financial assistance was provided by The Faculty of Pharmaceutical Sciences and Sanofi Winthrop, Canada. This research was supported by The Medical Research Council of Canada.
I. INTRODUCTION

The catastrophic release of methyl isocyanate (MIC) in Bhopal, 1984, resulted in massive human exposure to the toxic effects of this compound (Heylin, 1985), and subsequently much interest has been focussed on the deleterious impact of isocyanates on biological systems (Dagani, 1985). Under less severe circumstances, the incidence of human exposure to isocyanates occurs either through low-level industrial contact with the isocyanate itself (Vandenplas et al., 1993), or through precursors of the isocyanate. Both nitrosoureas (Colvin and Chabner, 1990) and formamides (Gescher, 1993) have the potential to generate isocyanates, and this is the commonality which unifies the study of these two apparently disparate groups of xenobiotics. Toxic episodes with nitrosoureas (Reed, 1985) and formamides (Mraz et al., 1989) have been associated with their carbamoylating activity, and so the study of these compounds as precursors of isocyanates provided the basis for this research.

Provided as a backdrop for the presentation of this thesis, relevant aspects of the research will be reviewed in this section. [1] Isocyanates, the unifying theme of this research on nitrosoureas and formamides, will be examined from the perspective of their electrophilic chemistry and their interaction with biological nucleophiles. [2] Nitrosoureas will be discussed with respect to their therapeutic application, metabolism and toxicology. [3] The etiology of formamide toxicity will be viewed in humans and animal models. [4] Cytochromes P450 are involved in the metabolism of nitrosoureas and formamides, and so a survey of this group of enzymes is warranted, especially with respect to catalysis, induction, inhibition, and relative distribution in microsomes and mitochondria. [5] The metabolism of isocyanates is closely associated with glutathione, and it is appropriate that the biological chemistry of this important thiol be examined with an emphasis on its role as a physiological detoxicant. [6] Atmospheric pressure ionization mass spectrometry constitutes a integral part of the methodology of this dissertation and accordingly the principles involved in this analytical
technique will be reviewed. [7] Topics presented in the foregoing preamble will bring into focus the specific objectives of our research on the metabolism and toxicology of nitrosoureas and formamides.

The names of several of the chemical compounds in this report are abbreviated, so for convenience, a listing of those which frequently appear is provided as an Appendix.

1. The Chemistry of Isocyanates

Isocyanates (R-N=C=O) are intrinsically quite reactive compounds and so, an understanding of their metabolism and toxicology is intimately associated with a knowledge of their chemistry. In the isocyanate group carbon adopts \( sp \)-hybridization. Carbon is bonded to nitrogen and oxygen through \( \sigma \)-orbitals along the \(-N-C-O\) axis, and through \( \pi \)-orbitals perpendicularly displaced to the central axis and to each other. As a result, the \(-N=C=O\) chain is approximately linear (Hargittai and Paul, 1977) with minimal delocalization of its \( \pi \)-electrons into conjugated systems (Entelis and Nesterov, 1966; Giles, 1977).

Internal conjugation within the isocyanate group gives rise to tautomeric structures with charge separation over the carbon, nitrogen and oxygen centres (Giles, 1977) (Figure 1). Tautomeric contributions to the isocyanate function decrease in the following order: structure a > structure c >> structure d, with all species serving to reduce the electron density at the carbon centre (Entelis and Nesterov, 1966). As a consequence, isocyanates readily undergo addition with nucleophiles, among which reactivity varies in the sequence \( R_2NH > RNH_2 > ROH > H_2O > ArOH > RSH \) (Arnold et al., 1957; Entelis and Nesterov, 1966). One of the possible mechanisms of addition is illustrated in Scheme 1. The alcohol and isocyanate exist in equilibrium with a \( sp^2 \)-hybridized-carbon-centered ionic complex which either undergoes (A) unimolecular prototropic rearrangement or (B) bimolecular reaction with a second \( R'OH \) molecule (Entelis and Nesterov, 1966).
Figure 1. Resonance forms contributing to the isocyanate function.

The reaction of isocyanates with amino, hydroxy and thiol groups in biological molecules is strongly influenced by molecular environment (Cohen and Oppenheimer, 1977). For example, N-carbamoylation of primary amines in the presence of vicinal carboxylic groups (e.g. α-amino acids) is followed by rearrangement to form hydantoins. In another instance, S-carbamoylation of glutathione (GSH) is favoured over N-carbamoylation (Stark et al., 1960), in an apparent reversal of the order of nucleophile reactivity described above (Arnold et al., 1957; Entelis and Nesterov, 1966). The higher reactivity of the thiol over the primary amine in GSH is probably a consequence of the formation of the more nucleophilic thiolate anion under physiological conditions. The carbamoylating activity of isocyanates may be modulated by the active site of enzymes. (1) O-Carbamoylation of the serine carbinol is sluggish for the free amino acid (Cohen and Oppenheimer, 1977); however, the reaction proceeds readily when the amino acid is present as a residue in chymotrypsin (Shaw et al., 1964; Babson et al., 1977). (2) Butyl isocyanate inactivates both chymotrypsin and elastase, whereas octyl isocyanate, because of its higher steric demand, inactivates only the former (Brown and Wold, 1973). (3) S-Carbamoylation of an active site cysteine in papain was 3,000-fold faster than the free amino acid (Sluyterman, 1967).
2. Nitrosoureas

Nitrosoureas emerged as a class of chemotherapeutic agents following indications of antitumor activity with methylnitrosoguanidine, and this group has subsequently flourished into a viable component in the battery of anticancer drugs (Colvin and Chabner, 1990). 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was the first of this class of drugs to undergo clinical trial, and has since been found useful in the treatment of brain tumors (Mitchell and Schein, 1986). Subsequent to its demonstrated efficacy in the cure of L1210 leukemia cells in mice (Johnston et al., 1966), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) has been constructively applied in the therapy of multiple myeloma (Finnish Leukaemia Group, 1992), advanced Hodgkin's disease (Koza et al., 1991; Szántó et al., 1991), and melanoma (Koller et al., 1994). Nitrosoureas will be reviewed with respect to their chemistry, metabolism and toxicology, with particular emphasis on CCNU and BCNU.

2.1. Chemistry

The stability of nitrosoureas in buffered aqueous solution is pH dependent and is optimal in the region pH 4 to 5 (Weinkam and Lin, 1982). At pH < 2 acid-catalyzed denitrosation affords nitrous acid and the urea. Base-catalyzed decomposition (pH > 5) as shown in Scheme 2 affords an initial diazohydroxide and an isocyanate which subsequently
decompose into a variety of compounds (Colvin et al., 1976; Colvin and Brundrett, 1981). Chemical decomposition of nitrosoureas proceeds at higher rates in serum compared to aqueous phosphate buffer (pH 7.4, 37°C), for BCNU ($t_{1/2}$ serum 14 min v.s. $t_{1/2}$ buffer 49 min) and CCNU ($t_{1/2}$ serum 30 min v.s. $t_{1/2}$ buffer 53 min) (Weinkam et al., 1980). A non-specific nitrosourea-serum protein interaction has been proposed to account for this catalytic effect (Weinkam and Lin, 1982).

\[
\begin{align*}
\text{Cl}_{\text{N}=\text{O}} \quad \text{N} \quad \text{N} \quad \text{R} & \quad \text{OH}^- \\
\end{align*}
\]

\[
\begin{align*}
\text{Cl}_{\text{N}=\text{C}} \quad \text{O} \quad \text{H} & \\
\text{R-NH-COOH} & \\
\text{RNH}_2 & \text{CO}_2
\end{align*}
\]

\[R = \text{Cl} \quad \text{BCNU} \]

\[\text{CCNU}\]

Scheme 2. Chemical decomposition of nitrosoureas. Adapted from (Colvin and Brundrett, 1981).

2.2. Mechanism of action

The alkylating and carbamoylating species released upon nitrosourea decomposition elicit extensive modifications to nucleic acids and enzymes (Colvin and Chabner, 1990). Studies by Cheng et al. (1972) on the interaction of CCNU with proteins and nucleic acids revealed that the former were extensively carbamoylated whereas the latter were preferentially
alkylated. Cytosine and guanosine residues are particularly vulnerable to nitrosourea alkylation (Weinkam and Lin, 1982), and it has been proposed that the structure of the intact nitrosourea ensemble confers some selectivity to the covalent modification of specific bases in nucleic acids (Naghipur et al., 1990).

Accumulating evidence suggests that the alkylation of DNA is responsible for the cytotoxicity of nitrosoureas (Weinkam and Lin, 1982). The cross-linking (Kohn, 1977; Ali-Osman, 1989), susceptibility to single-strand breaks (Lown and McLaughlin, 1979) and resistance to repair (Erickson et al., 1980) of nitrosourea-modified DNA are findings which substantiate this hypothesis. It is interesting to note, however, that carbamoylation could yet play some role in the expression of cytotoxic action, through the inhibition of RNA processing enzymes (Kann et al., 1974b), DNA processing enzymes (Kann et al., 1974a) and DNA polymerase II (Baril et al., 1975).

2.3. Metabolism

Nitrosoureas and their decomposition products are metabolized by cytochrome P450 (P450), NADPH-P450 reductase and glutathione-S-transferase (GST) (Scheme 3). P450 catalyzes the oxidative denitrosation of BCNU to the chemically stable, pharmacologically inert 1,3-bis(2-chloroethyl)urea (Hill et al., 1975). P450 2B1/2B2 has been implicated in the denitrosation process, with compelling evidence derived from studies in rat hepatic microsomes probed with anti-P450 2B1 IgG, as well as a reconstituted system with purified P450 2B1 (Weber and Waxman, 1993) (see Introduction/Section 4 for details on P450). Interestingly, P450 does not appear to metabolize CCNU in this fashion, but instead directs its activity toward alicyclic hydroxylation (May et al., 1974; Hill et al., 1975). Indeed, P450 mediates the microsomal hydroxylation of CCNU at several ring positions in a fashion dependent on treatment with inducers (Scheme 3, Table 1). Although reports vary on the extent to which each position is hydroxylated, trans-3- and cis-4-hydroxy CCNU constitute major microsomal metabolites and, in the event of phenobarbital (PB) treatment, cis-4-
hydroxy CCNU is predominant. Consistent with the pattern of hydroxylation obtained in microsomes, trans-2-hydroxy-, cis-3-hydroxy-, trans-3-hydroxy, cis-4-hydroxy- and trans-4-hydroxy CCNU were also identified in the plasma of rats dosed with CCNU, with cis-4-hydroxy-CCNU predominating with PB-pretreatment (Hilton and Walker, 1975b). In the case of human metabolism, CCNU undergoes less extensive ring hydroxylation compared to the rat, and only cis-4-hydroxy- and trans-4-hydroxy CCNU were identified in the plasma of patients receiving CCNU (Hilton and Walker, 1975a).

NADPH-P450 reductase, the electron donor to P450 (see Introduction/Section 4) plays a role in the metabolism of CCNU (Scheme 3) (Potter and Reed, 1982). Evidence suggests that the mechanism involves a one-electron reduction to afford the urea and nitric oxide (Potter and Reed, 1983). In the case of BCNU, NADPH-P450-reductase contributes negligibly to the denitrosation process (ca 3 %) without the recruitment of P450 monooxygenase activity (vide supra) (Weber and Waxman, 1993).

The denitrosation of BCNU is also supported by the phase II metabolism of glutathione S-transferase (GST) (See Introduction/Section 5.4.) as evidenced in the conversion of the nitrosourea to 1-chloroethyl-3-glutathionyl urea in the presence of rat liver cytosol (Scheme 3) (Hill, 1976). Also evident in these experiments was the fact that CCNU does not serve as a substrate for this biotransformation. It has since been established that cytoplasmic mu-class isozymes, particularly 3-4 and 4-4, are responsible for catalyzing BCNU denitrosation (Smith et al., 1989). Recently a role has also been demonstrated for microsomal GST in the denitrosation process, albeit at a lower rate than its cytoplasmic counterparts (Weber and Waxman, 1993).

A role for GSH (or cysteine) in the detoxification of the 2-chloroethyl carbocation was proposed for BCNU with the identification of thiodiacetic acid and S-carboxymethylcysteine as urinary metabolites in rats (Scheme 3) (Reed and May, 1975). It is possible that these metabolites resulted from the S-alkylation of GSH, followed by a combination of events
involving mercapturate pathway metabolism (see Introduction/Section 5.3) and hydrolysis/oxidation of the 2-chloroethyl substituent (Reed, 1981).

The *in vivo* formation of isocyanates derived from CCNU and its phase I metabolites has been inferred from the identification of cyclohexylamine and its hydroxylated analogues as metabolites of CCNU (Scheme 3) (Hilton and Walker, 1975b; Kohlhepp *et al.*, 1981). Evidence for the *in vivo* action of CCNU-derived isocyanates towards peptides has also come to the fore, with the discovery of four radiolabelled peptide conjugates as urinary metabolites of ring-labelled CCNU (Kohlhepp *et al*., 1981). Although it was determined that the molecular weights of the conjugates ranged from 243 to 629 a.m.u., the identity of these conjugates was not uncovered.
Scheme 3. Summary of the metabolism of BCNU and CCNU. See text for supporting references. (1) P450 2B1/2B2; (2) GST mu 4-4; (3) Chemical decomposition; (4) GSH (or cysteine) dependent metabolism; (5) Carbamoylation products - Unidentified; (6) NADPH-P450-reductase; (7) P450 alicyclic hydroxylation at designated sites (see Table 1); Decomposition to (8) isocyanate and (9) corresponding amine; (10) Carbamoylation products - Unidentified. CCNU, R = C6H11; BCNU, R = CH2CH2Cl.
Table 1. Profiles of P450 hydroxylation of CCNU in rat hepatic microsomes. Values listed are % composition of hydroxylated metabolites. Substrate metabolism was consistently higher in microsomes obtained from phenobarbital (PB) treated rats (Hilton and Walker, 1975b; May et al., 1975). Treatment of rats with 3-methylcholanthrene (3-MC) did not result in an elevated rate of CCNU microsomal metabolism. (ND = Not detected).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>trans-2- (%)</th>
<th>cis-3- (%)</th>
<th>trans-3- (%)</th>
<th>cis-4- (%)</th>
<th>trans-4- (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>ND</td>
<td>30</td>
<td>39</td>
<td>21</td>
<td>9</td>
<td>May et al., 1975</td>
</tr>
<tr>
<td>PB</td>
<td>ND</td>
<td>16</td>
<td>13</td>
<td>67</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3-MC</td>
<td>ND</td>
<td>25</td>
<td>40</td>
<td>30</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>14</td>
<td>trace</td>
<td>31</td>
<td>53</td>
<td>3</td>
<td>Hilton and Walker, 1975b</td>
</tr>
<tr>
<td>PB</td>
<td>3</td>
<td>3</td>
<td>11</td>
<td>77</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>PB</td>
<td>2</td>
<td>20</td>
<td>6</td>
<td>63</td>
<td>6</td>
<td>Farmer et al., 1978</td>
</tr>
</tbody>
</table>

2.4. Toxicity of nitrosoureas

The therapeutic usefulness of chronically administered nitrosoureas is considerably restricted by the occurrence of hematopoetic suppression, renal failure and hepatic damage (Johnston and Montgomery, 1986; Colvin and Chabner, 1990). A concern which has rapidly achieved prominence is the delayed and often irreversible condition of pulmonary fibrosis with BCNU (O'Driscoll et al., 1990; Hasleton et al., 1991; Taylor et al., 1991), and to a lesser extent CCNU (Stone and Richardson, 1987) and chlorozotocin (Smith, 1989). Review of this condition by Weiss et al. (1981) revealed an incidence of as high as 20 - 30% in the patient population for BCNU. Predisposing factors include pre-existing pulmonary disease, thoracic irradiation, oxygen therapy and concomitant medication with other pulmonary toxic agents. The mechanism by which BCNU elicits pulmonary toxicity is unclear (Smith, 1989); however, mounting evidence implicates the carbamoylating activity of the nitrosourea toward glutathione reductase in the lung (Stahl and Eisenbrand, 1991), particularly in the alveolar type II cell population (Smith et al., 1986; Jenkinson et al., 1994). Alveolar type II cells are responsible for producing pulmonary surfactant, a lipid-protein complex, and it is a suppressed production of the latter in BCNU-treated animals that is associated with the precipitation of widespread pulmonary lesions (Schuller et al., 1985).
3. Formamides

Human exposure to formamides usually occurs within laboratory or industrial settings, since these compounds, primarily N,N-dimethylformamide (DMF) and N-methylformamide (NMF) (Figure 2), are routinely employed as solvents. The topics of formamide toxicity and metabolism will be addressed here because accumulating evidence strongly suggests that toxic sequelae associated with formamides occur as a consequence of their metabolism (Gescher, 1993; Van den Bulcke et al., 1994).

![Figure 2. Structures of formamide compounds discussed in the thesis.](image)

3.1. Formamide exposure and toxicity

DMF is a dipolar aprotic solvent which is commonly used in the polymer industry. Toxicity in workers occupationally exposed to DMF has been documented as episodes of gastric irritation, nausea and hepatic damage (Redlich et al., 1990; Wang, J.D. et al., 1991). Incidence of human toxicity with the secondary formamide, N-methylformamide (NMF), stems primarily from its use in cancer chemotherapy. Contingent upon its demonstration of cytostatic activity in experimental tumors (Furst et al., 1955), NMF was used in phase I clinical trials in the treatment of prostatic, cervical and ovarian carcinomas (McVie et al.,
Despite initial signs of antineoplastic effectiveness, episodes of hepatotoxicity became increasingly apparent during phase II clinical trials (Eisenhauer et al., 1986). Consequently, the drug was considered unacceptable for therapeutic use.

Interest in our laboratory in the high molecular weight tertiary formamide N-methyl-N-(1-methyl-3,3-diphenylpropyl)formamide was borne out of the discovery of this compound as a metabolite of the anticholinergic aliphatic amine 3-dimethylamino-1,1-diphenylbutane (recipavrin) in rats (Slatter et al., 1990). We subsequently became aware that the tertiary formamide was a metabolic precursor of the secondary formamide, N-(3,3-diphenylpropyl)formamide (Slatter et al., 1989), which when administered as the parent drug, resulted in toxic episodes of biliary bleeding (Mutlib et al., 1990). Further support for our interest in high molecular weight formamides is drawn from the occurrence of N-formyl amphetamine (NFA), N-formyl methamphetamine and N-formyl 3,4-methylenedioxyamphetamine as significant contaminants in illicit preparations of amphetamine and its analogues (Figure 2) (LeBelle et al., 1973; Frank, 1983; Renton et al., 1993). The incidence of formamide contamination in these illegal drugs, which in 1981 constituted some 60 % of the production of clandestine laboratories (Frank, 1983), suggests the possibility of extensive human toxicity beyond the abuse potential (Renton et al., 1993)

3.2. Metabolism. A probe into the mechanism of formamide toxicity

Diverse strategies have been employed to uncover the mechanism of formamide hepatotoxicity. Early studies on the metabolism of NMF in mice suggested an association between its metabolism and GSH. Thus, it was observed that hepatotoxicity occurred coincidentally with the consumption of hepatic GSH and was exacerbated by prior depletion of hepatic GSH with butathionine sulfoximine (Pearson et al., 1987a). Furthermore, NMF became covalently bound to hepatic proteins in dosed mice and in microsomal incubations with the formamide as substrate (Pearson et al., 1987b). Covalent binding to microsomes was dependent on NADPH and reduced in the presence of glutathione (GSH). These lines of
evidence led to the conjecture that NMF was being metabolized to the actual offending agent methyl isocyanate (MIC) (Scheme 4).

Support was lent to this hypothesis with the identification of \( N \)-acetyl-S-(methylcarbamoyl)cysteine (AMCC) (Kestell et al., 1986) and S-(methylcarbamoyl)glutathione (SMG) (Threadgill et al., 1987) as urinary and biliary metabolites, respectively, of NMF. This metabolite profile was rationalized by NMF being oxidized to MIC which undergoes nucleophilic addition with GSH to form SMG. SMG is in turn metabolized by the mercapturic acid pathway to the \( N \)-acetylcysteine (NAC) conjugate (See Introduction/Section 5 for details on GSH metabolism) (Scheme 4). The unstable nature of MIC does not permit its detection as a metabolite of NMF, and so the intermediacy of the isocyanate is speculative. However, the identification of SMG (Pearson et al., 1990a) and AMCC (Slatter et al., 1991) as metabolites of rats dosed with MIC itself speak in favour of this compound being a biotransformation intermediary of NMF.
Scheme 4. Metabolic pathways for the formamides DMF (Kestell et al., 1985; Gescher, 1993) and N-methyl-N-(1-methyl-3,3-diphenylpropyl)formamide (Slatter et al., 1989; Mutlib et al., 1990). CH$_3$NH$_2$ and CO$_2$ were characterized as metabolites of NMF.

The finding that the microsomal metabolism of NMF was dependent on NADPH suggested the mediation of P450 catalysis, and work by Cross et al. (1990) has solidified this contention. It was observed that in addition to its dependence on NADPH, this reaction was inhibited by carbon monoxide and by SKF 525-A, a broad spectrum inhibitor of P450 activity (See Introduction/Section 4 for details on P450). Efforts to decipher the isozymic involvement in the bioactivation of NMF to MIC have led to P450 2E1 as the primary candidate (Hyland et al., 1992). This argument was based on evidence that the microsome-catalyzed reaction was induced by acetone and inhibited by anti-P450 2E1 IgG and diethyldithiocarbamate (DEDTC), a selective inhibitor of P450 2E1 (See Introduction/Section 4 for details on P450). Although the P450 2E1 mediation of NMF oxidation to MIC is
established, the mechanistic details on the reaction are sparse. It does appear, however, that formyl hydrogen abstraction is the rate-determining step based on the primary kinetic isotope effect associated with the formation of SMG and AMCC from the isotopomer CH$_3$NCO[²H] in mice (Threadgill et al., 1987).

Despite the interest shown in the metabolism of DMF and NMF, few studies in the literature have focused on large formamides (Mutlib et al., 1990; 1991). Present evidence suggests that some parallels in metabolism exist between the low and high molecular weight formamides (Scheme 4). Thus, N-methyl-N-(1-methyl-3,3-diphenylpropyl)formamide is metabolized in the rat to a hydroxymethyl derivative (Mutlib and Abbott, 1992) and a secondary formamide (Slatter et al., 1989). The secondary formamide is in turn bioactivated to a putative isocyanate, which although not detected, has been inferentially proposed from downstream GSH-dependent metabolites (Mutlib et al., 1990). This evidence suggests that isocyanates could mediate the toxicity of high molecular weight formamides, and given the forensic relevance of these compounds (vide supra), their metabolism is indeed deserving of further attention.

4. Cytochrome P450

The cytochromes P450 (EC 1.14.14.1), so designated because of the absorbance wavelength ($\lambda_{max} = 450$ nm) of the CO difference spectrum of the reduced enzyme (Omura and Sato, 1964), constitute a family of hemoproteins which command a central role in phase I metabolism. Although the family is characterized by a thiolate-bound heme prosthetic group and an activated oxygen mechanism of catalysis, there exists among cytochromes P450 a diverse array of apoenzyme structures which is manifest in a plethora of substrate specificities for compounds of endogenous and xenobiotic origin (Soucek and Gut, 1992).
4.1. The nomenclature of cytochromes P450

Because of the diversity of P450 genes, a nomenclature system was implemented to systematize genetic classification (Nebert et al., 1987). Since then, the rules governing classification have evolved to keep pace of this rapidly expanding field with the most recent update describing some 221 P450 genes (Nelson et al., 1993). In this thesis, the rules of nomenclature proposed in the latest report (Nelson et al., 1993) will be adopted for genes and gene products which have been characterized to date. By way of example, consider the gene CYP2E1 and its product P450 2E1. Denotations for the gene are root symbol "CYP", family "2", subfamily "E" and individual gene "1". Denotations for the gene product are likewise denoted except the root symbol is designated "P450" instead of "CYP". Gene products for which the gene has not been characterized are named according to the citing author e.g. P450mt1, P450mt2, P450mt3 and P450mt4 (Shayiq and Avadhani, 1990). There exists considerable amino acid sequence homology (≥ 97 %) in the P450 2B family for P450 2B1 and 2B2 (Ryan and Levin, 1990) and in the P450 3A family for P450 3A3 and 3A4 (Wrighton and Stevens, 1992). In situations were the P450 2B and P450 3A proteins are not distinguished, the collective terms P450 2B1/2B2 and P450 3A3/3A4, respectively, will be used.

4.2. Mechanism of microsomal P450 catalysis

During the course of P450 oxidative catalysis, endogenous compounds (steroids and fatty acids) and xenobiotics (drugs and environmental pollutants) are rendered more hydrophilic in preparation for elimination. One of the distinguishing features of P450 catalysis is the involvement of activated oxygen. During the course of one P450 catalytic cycle, molecular oxygen dissociates between substrate oxidation and water formation (Ortiz de Montellano, 1986). This mechanism can be envisioned in Scheme 5 (Guengerich and Macdonald, 1992).
Considered in a stepwise fashion, (1) binding of substrate near the distal ligand site of ferric P450 facilitates (2) the first one electron reduction with NADPH as the donor. This reaction is catalyzed by the flavoprotein NADPH-P450 reductase with the transfer of two electrons in singular fashion to P450 (Peterson and Prough, 1986; Backes, 1993). In the sequence of steps (3 - 4) the ferrous P450-substrate complex binds dioxygen to afford the unstable [Fe$^{II}$O$_2$] species which undergoes the second one electron reduction. In step (5) dioxygen atoms dissociate between insertion into the activated P450[FeO]$^{3+}$-substrate complex and into a molecule of water (Ortiz de Montellano, 1986). In the conclusion of the catalytic cycle (steps 6 - 7), P450[FeO]$^{3+}$ supports the insertion of oxygen into the substrate to afford the hydroxylated product (P) which is released from the enzyme (Guengerich, 1990).

Scheme 5. Overall pathway for P450-mediated catalysis involving (1) substrate (S) binding, (2) first one electron reduction, (3) dioxygen binding, (4) second one electron reduction, (5) dioxygen cleavage, (6) product (P) formation, and (7) product dissociation (Adapted from Guengerich and Macdonald, 1992).
4.3. Cytochrome P450 induction and inhibition

Induction and inhibition are two processes which are known to preferentially modulate the expression of P450 activity, and accordingly these techniques have been exploited as probes into the isozymic involvement in oxidative biotransformations. Whereas P450 induction occurs in response to a genetic message and is an obligate property of the intact cell, P450 inhibition is elicited either in vivo, in intact cells or in subcellular fractions.

4.3.1. Cytochrome P450 induction

The term "induction" is familiarly viewed as the elevated expression of a particular isozyme (or group of isozymes) which is elicited by a xenobiotic species or a perturbation of the physiological state. Several compounds are now known to act as inducers as typified in Table 2 which provides an overview of some agents discussed in this dissertation. Table 2 is illustrative of three salient features of P450 inducers and the isozymes which they modulate. First, several isozymes (P450 2B1, 2B2 and 2E1) may be induced by the same agent (acetone); second, one particular isozyme (P450 2E1) may be induced by a variety of compounds (acetone, ethanol and isoniazid); and third, one agent (phenobarbital) may induce different isozymes in different species - P450 2B1/2B2 (rat) and P450 3A3/3A4 (human).

Table 2. Inducible hepatic microsomal cytochromes P450.

<table>
<thead>
<tr>
<th>P450 isozyme</th>
<th>Inducer</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>2E1</td>
<td>acetone</td>
<td>Johansson et al., 1988</td>
</tr>
<tr>
<td></td>
<td>ethanol isoniazid</td>
<td>Thomas et al., 1987 Thomas et al., 1987</td>
</tr>
<tr>
<td>3A1/3A2</td>
<td>dexamethasone</td>
<td>Cooper et al., 1993</td>
</tr>
<tr>
<td>3A3/3A4</td>
<td>phenobarbital</td>
<td>Wrighton and Stevens, 1992</td>
</tr>
<tr>
<td>mt1 and mt2</td>
<td>β-naphthoflavone</td>
<td>Raza and Avadhani, 1988</td>
</tr>
<tr>
<td>mt3</td>
<td>phenobarbital</td>
<td>Shayiq and Avadhani, 1989</td>
</tr>
<tr>
<td>mt4</td>
<td>phenobarbital</td>
<td>Shayiq and Avadhani, 1990</td>
</tr>
</tbody>
</table>
Diverse mechanisms by which P450 inducers elicit their activity have been characterized, including upregulation of the encoding CYP gene, mRNA stabilization and attenuated P450 degradation (Okey, 1990). The sequence of events involved in P450 induction is best understood for P450 1A1 and 1A2 by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). It is now generally accepted that the mechanistic sequence involves (1) binding of the inducer species to a cytosolic receptor, (2) import of the receptor-inducer complex into the nucleus, and (3) association of the complex with the encoding CYP1A1 and 1A2 genes which ultimately triggers an increase in the translation of the corresponding P450 proteins (Okey et al., 1994).

Phenobarbital (PB) induction of P450 2B1 and 2B2 also appears to be transcriptionally dependent; however, the events which occur upstream of transcription remain obscure (Waxman and Azaroff, 1992). Because of the diverse structural nature of PB-like inducers, the identification of a PB receptor has as yet eluded researchers, and it is conjectured that should such a hypothetical receptor exist, complexation with its ligands would necessitate either a "sloppy fit" or receptor "plasticity" (Okey, 1990). Although P450 2B1/2B2 and 2E1 belong to the same P450 2 family, P450 2E1 does not appear to be modulated by PB-like inducers. Instead, this isozyme appears to be induced by solvents (acetone, ethanol, trichloroethylene) and some involatile compounds (isoniazid, pyrazole) (Koop et al., 1985). These inducers appear to modulate the cascade to the P450 2E1 gene product either at the level of increased transcription, mRNA stabilization or protein stabilization (Porter and Coon, 1991). Although the role of transcriptional activation of CYP2E1 by chemical agents has been downplayed in favour of protein stabilization (Song et al., 1989), recent evidence supports the former mechanism as a factor to also be considered for induction (Kraner et al., 1993; Takahashi et al., 1993).
4.3.2. Cytochrome P450 inhibition

A diverse array of inhibitory agents now exist which are capable of undermining the catalytic performance of P450 isozymes, either by targeting elements of the prosthetic ensemble or the apoenzyme structure itself. Of these agents, reversible and mechanism-based inhibitors will be considered briefly here (Figure 3).

![Chemical structures](image)

**Figure 3.** Structures of reversible (metyrapone) and mechanism-based (orphenadrine and DEDTC) inhibitors of cytochrome P450.

Reversible inhibitors block the initiation of the P450 oxidative cycle because they compete with other substrates for occupancy of the catalytic site by either binding to the hydrophobic apoenzyme domain or complexing prosthetic heme iron (Ortiz de Montellano and Reich, 1986). Metyrapone, by virtue of its aromatic nuclei and pyridyl nitrogen atoms, accomplishes each of these respective events simultaneously, and as such its efficiency as an inhibitor is synergistically enhanced. One of the characteristic features of reversible inhibitors is their rather broad spectrum of activity which arises because recognition of the inhibitor as a substrate is not isozyme specific. Notwithstanding, in the case of metyrapone, there have been reported instances of preferential inhibition of P450 isozymes (Goeptar *et al.*, 1993).

Mechanism-based inhibitors exert their influence during the course of the P450 catalytic cycle. Accordingly, the inhibition event in this case is contingent upon not only recognition of the inhibitor as a substrate, but its enzyme-mediated transformation as well. Reactive species formed during the course of P450 catalysis target vulnerable structures on
the isozyme, thereby conferring some degree of selectivity to mechanism-based inhibition (Ortiz de Montellano and Reich, 1986).

Metabolite-intermediate (MI) complexation (e.g. orphenadrine) and covalent apoprotein binding (e.g. DEDTC) are two processes involved in catalytic inhibition. Orphenadrine inhibition tends to be selective for P450 2B1/2B2 and is thought to be mediated through activation of the tertiary amine to a nitroso metabolite (Murray and Reidy, 1990). Thus, in a combination of events involving N-demethylation, N-hydroxylation and N-oxidation, a metabolite intermediate of orphenadrine is generated, and forms a stable complex with the ferrous heme of P450 2B1/2B2. As conceded in a recent review, there is little known about the selectivity of MI complexation agents (Murray, 1992). Diethyldithiocarbamate (DEDTC) is typical of a class of thiol compounds which, upon activation to an oxidized sulfur species, covalently bind to the P450 apoenzyme (Ortiz de Montellano and Reich, 1986; Murray and Reidy, 1990) and convert P450 to the inactive P420 form (Miller et al., 1983). DEDTC preferentially inhibits P450 2E1 (Lauriault et al., 1992), although the mechanistic basis for its isozyme-selective inhibition remains unclear.

4.4. Mitochondrial cytochromes P450

Mammalian mitochondrial P450 was first characterized in adrenal cortex and since then the distribution of these P450s has been recognized in steroidogenic organs, kidney and liver (Omura, 1993). Although a long standing association exists between mitochondrial P450 and endogenous substrates (Ghazarian and DeLuca, 1974; Jefcoate, 1986), it is now recognized that P450 isoforms from this organelle metabolize xenobiotic compounds and are induced by chemical agents (Table 2) (Shayiq et al., 1991). Fundamental differences have been identified between mitochondrial P450 and their endoplasmic reticular (microsomal) counterparts. Prominent among these distinguishing features are (1) the operations involved in posttranslational processing of the P450 gene products (Jansson, 1993), and (2) the mechanisms involved in electron-transfer to P450 (Jefcoate, 1986).
4.4.1. Protein import into mitochondria

Both microsomal and mitochondrial cytochromes P450 are encoded on nuclear DNA and synthesized in the cytoplasm. However, whereas microsomal P450s are cotranslationally inserted in mature form into the endoplasmic reticulum (Black, 1992), mitochondrial P450s are translated as a soluble precursor species scheduled for further processing before the product is finally internalized at its mitochondrial destination (Ogishima et al., 1985). Precursor proteins destined for the mitochondria generally incorporate a hydrophobic N-terminal presequence which contains information necessary for their import and localization (Kumamoto et al., 1989). Of the four mitochondrial compartments, namely the outer membrane, intermembrane space, inner membrane and matrix, P450s occupy the inner membrane with minor domains extending into the matrix (Lombardo et al., 1986). Given the stringency with which this organelle regulates the transit of molecular species, several operations are involved in the import of the precursor (Schatz, 1993). In essence, the precursor, complexed with a cytosolic "chaperone" protein is recognized by mitochondrial receptors which channel the unaccompanied precursor to the reception of a matrix-associated protein. Once the precursor is finally ushered into the matrix, the targeting presequence is cleaved by a local endoprotease, thereby facilitating anchorage of the mature protein and insertion of heme (Omura, 1993). It should be noted that the sorting mechanism, described here for mitochondrial P450s 11A1 and 11B1, addresses only the targeting of the inner membrane, and there exists a host of alternative mechanisms in the complex machinery of mitochondrial protein import (Pfanner and Neupert, 1990; Glick and Schatz, 1991).

4.4.2. Mechanism of mitochondrial P450 catalysis

NADPH synthesized in the cytoplasm to fuel microsomal P450 activity is not free to traverse the intact mitochondrial membrane (Jefcoate, 1986). Consequently, reducing equivalents required for localized P450 activity in mitochondria are of necessity generated in
situs by three enzymatic processes - NADP+\textendash NAD transhydrogenase, NADP+-isocitrate dehydrogenase, and NADP+-linked malic enzyme.

In the shuttle of electrons from NADPH to mitochondrial P450, isoforms from this organelle demonstrate an obligate dependence on a mitochondrial electron transport apparatus *viz* ferredoxin/NADPH-ferredoxin reductase or adrenodoxin/NADPH-adrenodoxin reductase (Jefcoate, 1986; Schenkman, 1993). In fact, this feature has been viewed as compelling evidence in favour of mitochondrial P450-mediated activity in reconstituted systems (Shayiq *et al.*, 1991). Thus, the catalysis of benzphetamine *N*-demethylation by purified P450mt3 (Shayiq and Avadhani, 1989) and P450mt4 (Shayiq and Avadhani, 1990) was operational in the presence of ferredoxin/NADPH-ferredoxin reductase but inactive in the presence of microsomal NADPH-P450-reductase.

5. **The role of glutathione in redox homeostasis and phase II metabolism**

The tripeptide thiol glutathione (GSH), \(\gamma\text{-L-glutamyl-L-cysteinylglycine}\), is ubiquitous in living systems and is structurally endowed to fulfill its role as a cellular detoxicant (DeLeve and Kaplowitz, 1991). Firstly, the \(\gamma\text{-glutamylcysteinyl}\) linkage renders GSH resistant to common peptidases, and accordingly, hydrolysis of the peptide is only initiated by the targeted action of \(\gamma\text{-glutamyltranspeptidase}\). Secondly, the redox potential (half-cell reaction \(E^{0}_{\text{GSSG/GSH}} = -0.26\), Millis *et al.*, 1993) and soft nucleophilic character (Coles, 1985) of the cysteinyl thiol are germane to the role of GSH as both an antioxidant and a "scavenger" of electrophilic compounds.

5.1. **Organ distribution, synthesis and compartmentalization of glutathione**

The distribution of GSH in mammalian organs tends to parallel the demand each system makes for the detoxifying action of the thiol. Thus, a rank order of GSH concentration from 7.30 to 2.90 \(\mu\text{mol/g-tissue}\) has been described in rats for liver > kidney > lung (Kretzschmar and Klinger, 1990). As the most active organ in oxidative metabolism the
liver is subject to a barrage of electrophilic species. Moreover, this organ and the kidney are crucial excretory centres, and are exposed to high concentrations of toxins. Inhalational exposure of the lung to oxygen, oxidant species and volatile xenobiotics is countered by high concentrations of GSH in the alveolar epithelium (Cantin et al., 1987).

Intracellular GSH is essentially distributed between the cytosol and mitochondria, each accounting for 85 % and 15 % of total non-protein thiol, respectively (Meredith and Reed, 1982). The synthesis of GSH in the cytoplasm is mediated by \( \gamma \)-glutamylcysteine synthetase and glutathione synthetase which sequentially catalyze the condensation of glutamate to cysteine and glycine in two ATP-dependent steps. The first step is rate limiting and regulated by feedback inhibition from GSH (Richman and Meister, 1975).

Mitochondrial GSH is not synthesized locally but is instead, imported from the cytoplasm (Griffith and Meister, 1985). A two-component transport system has been identified which serves to retain mitochondrial GSH at the expense of cytoplasmic reserves (Martensson et al., 1990). Despite the tidal exchange of GSH between mitochondria and cytosol, these pools are separate and distinct. Two lines of evidence speak for this partitioning. Mitochondrial and cytosolic compartments vary in their rate of GSH turnover (Meredith and Reed, 1982), and vary in their susceptibility to GSH depleting agents (Meredith and Reed, 1983; Shan et al., 1993). Mitochondrial GSH is crucial for cytoprotection, and depletion of this pool often precedes cell injury and eventually cell death (Reed, 1990).

5.2. The glutathione redox cycle

During the course of aerobic respiration electron leakage from the respiratory chain gives rise to \( O_2^- \) which dismutates to \( H_2O_2 \) (Chance et al., 1979). \( H_2O_2 \) is a potent oxidant; however, the hazard it presents is alleviated by the activity of GSH peroxidase (GP). GP is a component of the glutathione redox cycle which is operative in both mitochondria and cytosol (Scheme 6)(Reed, 1990). During the course of this cycle, GP catalyzes the reduction of \( H_2O_2 \)
to water with the attendant oxidation of GSH (reduced glutathione) to GSSG (oxidized glutathione). The oxidized thiol is in turn, reduced back to GSH by the action of glutathione reductase (GR). Thus, GR plays a key role in the maintenance of cellular redox homeostasis, a fact borne out in the low intracellular concentrations of GSSG in rat liver ([GSH]/[GSSG] \( \approx 22 \)) (Kosower and Kosower, 1978). The course of the glutathione redox cycle is fundamentally dependent on NADPH, and the enzyme activities which fuel this cofactor include isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate dehydrogenase (Reed, 1986).

![Scheme 6. The glutathione redox cycle. Adapted from Reed (1990).](image)

5.3. Conjugation of glutathione to electrophiles

The thiol moiety of GSH plays a key role in the conjugation and ultimate elimination of compounds which are either intrinsically reactive or so rendered by P450 bioactivation (Ketterer and Mulder, 1990). Nucleophilic attack of the GSH thiol at electron deficient centres on the substrate renders the latter more hydrophilic and consequently more readily able to access excretory pathways in the form of the thiol conjugate. GSH conjugates are generally excreted in bile or metabolized in the mercapturate pathway by the sequential activity of \( \gamma \)-glutamyltranspeptidase (\( \gamma \)-GT), peptidase and N-acetyltransferase (NAT), and excreted as the N-acetylcysteine (NAC) conjugates in urine (Tate, 1980). There exists a myriad of compounds which undergo GSH conjugation by either nucleophilic substitution, addition or addition-elimination mechanisms (Figure 4). Conjugation substrates include aliphatic, olefinic and aromatic halides, epoxides, enones, isothiocyanates and isocyanates.
Figure 4. Structures of compounds which undergo GSH conjugation. Arrows indicate sites of GSH thiol attack. Conjugation mechanisms involve substitution at alkyl halides and epoxides, addition-elimination at olefinic halides, and addition at enone and cumulated unsaturated systems.

Although GSH conjugation of electrophilic species is by and large a deactivation process, there are situations where toxicity is paradoxically amplified or delayed in the form of the thiol conjugate. This aspect of GSH conjugation was aptly brought into focus when reviewed by van Bladeren (1988), and has since gained considerable attention (Anders, 1990; Baillie and Slatter, 1991; Anders et al., 1992; Dekant, 1993). Three such situations are briefly addressed here (Scheme 7):

(A) Two successive $S_N2$ displacements of Br$^-$ from the 1- and 2-positions of 1,2-dibromochloropropane, first through attack of the GSH thiol and second through anchimeric assistance of the thioether, afford the episulfonium ion (Pearson et al., 1990b). Evidence suggests that the episulfonium ion could be involved in the toxicity and mutagenicity of the alkyl halide.

(B) Conjugation of GSH to the terminal position of hexachlorobutadiene (HCBD) by addition-elimination; followed by the sequential activity of $\gamma$-GT, peptidase and cysteine
conjugate β-lyase gives rise to the thioketenone which is considered the offending agent in nephrotoxicity (Dekant and Vamvakas, 1993).

(C) Nucleophilic addition of GSH to isocyanates (e.g. methyl isocyanate) affords the thiocarbamate conjugate (Pearson et al., 1990a). However, E1cB reversal of this process is facile under physiological conditions, which suggests that the conjugate could act as a vehicle for the toxic carbamoylating activity of the isocyanate (Baillie and Slatter, 1991; Pearson et al., 1991). Envisioned in this mechanism is the notion that isocyanates formed at one physiological location, upon binding to GSH, could be transported by biological fluids (e.g. bile and blood) in the form of the conjugate to distal sites where the isocyanate could be released upon equilibrium reversal. This proposed mechanism of toxicity is particularly relevant to the research in this thesis on the isocyanates derived from nitrosoureas and formamidines.

Scheme 7. Mechanisms of GSH-mediated xenobiotic toxicity: (A) episulfonium ion formation; (B) cysteine conjugate β-lyase bioactivation; and (C) equilibrium formation of a thiocarbamate.
5.4. Glutathione-S-transferases

The reactivity of the GSH with electrophiles varies considerably and depends on the character of the electrophile (Coles, 1985). Because the GSH thiol is a soft nucleophile with a diffuse, polarizable charge density, it favours soft electrophiles. Whereas the chemical reaction of GSH with isocyanates (soft electrophiles) proceeds readily, conjugation with epoxides (hard electrophiles) proceeds rather stubbornly. Conjugations of the latter type invariably necessitate the intervention of glutathione-S-transferase (GST) catalysis.

5.4.1. Nomenclature, compartmentalization and function of glutathione S-transferases

Glutathione-S-transferases (GSTs) (EC 2.5.1.18) comprise a family of multi-functional enzymes which are ubiquitously distributed in aerobic organisms. At least five classes - alpha, mu, pi, theta and microsomal - have been delineated on the basis of their immunological and structural attributes with considerable sequence overlap apparent between species. Recently, a mitochondrial GST immunochemically related to the cytosolic alpha class has been characterized (Addya et al., 1994). Cytosolic GSTs together constitute up to 10% of the protein in this pool, and consequently these isozymes have received most attention (van Bladeren and van Ommen, 1991). Although the principal function of GSTs resides in the catalysis of GSH conjugation to electrophiles, other important support functions have been identified, including peroxidase catalysis, transport of non-substrate ligands (e.g. heme, bile acids, steroids), and covalent scavenging of electrophilic species (DeLeve and Kaplowitz, 1991).

5.4.2. Mechanism of action of glutathione-S-transferases

During the course of GST catalysis, a diversity of hydrophobic substrates are conjugated to the GSH cosubstrate. Some degree of substrate specificity exists among GSTs; for example, in the rat mu class, isozyme 4-4 preferentially catalyzes GST conjugation to epoxides and enones, whereas 3-3 is partial to aromatic nucleophilic substitution (Zhang et
al., 1992). However, as a family, GSTs generally exhibit considerable overlapping substrate specificity. The mechanism of catalysis appears to reside on facilitating the approach of GSH, in its thiolate form, to the substrate in a desolvated environment (Armstrong, 1991).

Contact between the substrate and cosubstrate GSH is enabled by the close proximity of the binding sites for these ligands. The substrate binding domain of GSTs, consistent with the nature of its occupants, is generally hydrophobic, being so rendered by considerable contribution from C-terminal residues (Wilce and Parker, 1994). Binding specificity for GSH is high with some 21 potential interactions for the cosubstrate identified in the pi class GSTs (Wilce and Parker, 1994). The γ-glutamyl element of GSH and thiol orientation appear to be critical determinants for cosubstrate specificity (Adang et al., 1988). Interestingly, a recent report has identified N-acetylcysteine (NAC) as a cosubstrate for microsomal GST, and this property has been exploited to distinguish GST isozyme(s) associated with this subcellular fraction (Weinander et al., 1994). Critical to the catalysis of GSTs is the presence of a tyrosine residue near the N-terminal. Present as Tyr-7 in the pi class GSTs, this residue is strongly conserved in all known GSTs (Wilce and Parker, 1994). This residue appears to promote the formation of the thiolate by lowering the pKa of GSH from ca 9 to ca 6 (Chen et al., 1988). Evidence suggests that tyrosine facilitates thiolate formation by hydrogen bond donation to GSH, orienting GSH at the active site and enabling the exit of the thiol proton from the active site (Liu et al., 1992).

6. Atmospheric pressure ionization liquid chromatography/mass spectrometry (API LC/MS)

API LC/MS, the atmospheric pressure conjugation of high pressure liquid chromatography (HPLC) to mass spectrometry (MS) has evolved into a powerful synergism which has catalyzed the development in the analysis of biological molecules. HPLC provides chromatographic separation of components in complex mixtures, frequently without the necessity for derivatization, and tends to be compatible with biomolecules encompassing a
broad spectrum of thermal stability and molecular weight. Detection by MS complements HPLC separation by providing valuable information on the molecular structure of an analyte. API LC/MS constitutes an integral part of this research, and a brief review of the concepts involved in this analytical technique are reviewed here.

6.1. API Instrumentation

The principle of API MS resides on the separation of the atmospheric pressure ion source region from the high vacuum mass analyzer, and is accomplished in current designs by a minute orifice draped with a partitioning curtain of N₂ (Figure 5) (Huang et al., 1990). Gas phase ions (vide infra) drawn through the orifice by an electric field enter the high vacuum region and undergo free-jet expansion with concomitant adiabatic cooling. As a result, clustering of solvent and sample ions occurs. Ion focussing and ion acceleration processes occurring en route to the mass analyzer region destabilize clusters allowing extricated pseudomolecular ions to proceed to the mass analyzer. API confers specific advantages to the LC/MS interface. (1) Because ionization is accomplished at atmospheric pressure, a large population of analyte ions is sampled; and (2) the gas curtain interface facilitates an efficient transfer of ions from atmospheric to high vacuum regions.

Figure 5. Schematic representation of the API LC/MS interface showing the generation of gas phase ions at atmospheric pressure and their transit to the high vacuum mass analyzer region. (Adapted from Huang et al., 1990).
6.2. Gas phase ionization

Three common approaches to the generation of gas phase ions will be addressed here: atmospheric pressure chemical ionization (APCI), electrospray and ionspray. However, API remains an active area of mass spectrometry research and innovations, like ultrasonically assisted electrospray, are continually coming to the fore (Banks, Jr., et al., 1994).

In the process of APCI, HPLC eluate is conducted along a heated probe which converges at its tip with the coaxial flow of a nebulizer gas (Covey et al., 1986). As the eluate emerges from the probe it evaporates to afford a vapour of solvent and solute molecules. A corona discharge across the gas phase mixture promotes ionization of solvent molecules, which in turn ionize solute molecules for sampling by the mass analyzer. Among the API ion sources, APCI most readily tolerates the high flow rates (≤2 mL/min) employed in conventional HPLC systems (Covey et al., 1986), but is somewhat limited by the occurrence of thermally-associated analyte decomposition and fragmentation as a result of the relatively high vapour temperature (125-150 °C) (Avery et al., 1992; Kasuya et al., 1992).

The electrospray process depends on the fact that imparting charge to the surface of a solution as it traverses a high voltage (3,500 V) needle forms gaseous ions (Whitehouse et al., 1985). In principle, as the solution is charged and droplets contract due to solvent evaporation, further dispersion is effected due to Coulombic repulsion at the droplet surface. This event progresses until the droplet explodes at the approach of the Rayleigh limit - the point at which the repulsive forces due to surface charging are equivalent to the cohesive forces of surface tension. This cycle continues until field-induced effects become operative, ejecting solute ion clusters from the droplet surface into the gas phase. Electrospray provides an alternative to APCI and its inherent thermally-associated deficiencies (vide supra). Electrospray is unfortunately limited to low flow rates (5-200 μL/min) for optimal efficiency; however, adaptation to conventional flow HPLC systems can be effected by sample splitting without appreciable loss in sensitivity.
Figure 6. Diagrammatic representation of the electrospray ionization process. Charge is acquired by the solution droplet because of the potential difference across the electrospray needle. As solvent evaporates Coulombic repulsion shatters the droplet. This process continues until field-induced effects eventually expel ion clusters into the gaseous phase (Adapted from Fenn et al., 1989).

Ionspray, pneumatically-assisted electrospray, was borne out of efforts by Bruins and coworkers (1987) to further improve the performance of API MS. These researchers implemented a coaxial delivery of nebulizing gas to quench the attendant corona discharge emanating from the spray capillary during high voltage electrospray. It became apparent that pneumatic assistance confers several ancillary benefits to the electrospray process including improved nebulization, reduced clustering in free jet expansion stage, and an accommodation of higher effluent flow rates (≤1 mL/min) with increased aqueous composition. Ionspray MS has found useful application in the analysis of proteins (Covey et al., 1988) and peptides derived from tryptic digests (Covey et al., 1991).

6.3. Liquid chromatography/tandem mass spectrometry (LC/MS/MS)

The mild ionization conditions of LC/MS frequently retain the integrity of the pseudomolecular ion. Thus, in order to access further structural information, the pseudomolecular species is taken as a precursor to be fragmented into a family of product ions (Covey et al., 1986). In order to elevate the efficiency of product ion formation beyond a singular unimolecular decay process, interaction with a neutral target gas (e.g. argon) is
frequently employed in the process of collisionally activated dissociation (CAD). Two major parameters governing the fragmentation process include the collision energy and the collision gas pressure (Bean et al., 1991). In the triple stage quadrupole mass spectrometer used in the studies here, the configuration comprises two quadrupole mass filters (Q₁ and Q₃) with the central collision cell (Q₂) regulated only by radiofrequency voltage. The collision energy is a function of the potential difference between the ion source and Q₂. The collision gas pressure determines the frequency of target gas collision.

The utility of LC/MS/MS resides in the fact that both pseudomolecular and fragment ion information is simultaneously acquired. This facility affords an assortment of useful options in the identification and quantitation of xenobiotic metabolites. Some of these techniques include (1) selected reaction monitoring (SRM), (2) parent ion scanning and (3) neutral loss. In SRM, the Q₁ mass analyzer filters specified ions to Q₂, and from there only selected ions of the product ion population are sampled by Q₃. The benefit derived from this process, as opposed to sampling the entire fragment ion spectrum, is that the ion statistics are improved with an attendant increase in sensitivity. Judicious assignment of precursor/fragment combinations have afforded sensitive and specific quantitative analytical procedures in which chromatography is minimal (Gilbert et al., 1992) or entirely circumvented (Davis et al., 1993). In the parent ion scanning experiment, a fragment ion, often one which is diagnostic for a class of compounds, is selected during a chromatographic run and only the event of precursor ions giving rise to the selected fragment is recorded. Neutral loss is somewhat similar in principle to parent ion scanning. In this case, however, Q₁ and Q₃ mass analyzers are offset by a mass difference which corresponds to the loss of a diagnostic fragment. Because parent ion scanning and neutral loss record characteristic MS events, these techniques afford the opportunity to screen for designated classes of xenobiotic metabolites (Davis et al., 1993; Kassahun et al., 1994).
7. Thesis objectives

It is quite likely that some of the toxic sequelae associated with the nitrosoureas CCNU and BCNU (Introduction/Section 2.4), and the formamide NFA (Introduction/Section 3.2) are elicited by their involvement as precursors of isocyanates. It was hypothesized that in vivo, CCNU and its phase I metabolites could give rise to cyclohexyl isocyanate, 4-hydroxycyclohexyl isocyanate and 3-hydroxycyclohexyl isocyanate (Scheme 3); BCNU could decompose to 2-chloroethyl isocyanate (Scheme 3); and NFA could be bioactivated to 1-methyl-2-phenylethyl isocyanate (MPIC) (Scheme 4). The envisaged reaction of these isocyanates with GSH in vivo was viewed as an opportunity to investigate this event in the form of biliary GSH conjugates and urinary NAC conjugates as mercapturate pathway metabolites. A possible mechanism of toxification which was of considerable interest was the impact of isocyanates on mitochondrial GSH. Thus, it was relevant to this research to identify processes whereby these electrophiles could be introduced to this critical thiol pool, either through transport from the cytosol in the form of thiocarbamate conjugates (Introduction/Section 5.3) or by in situ bioactivation of formamides (Introduction/Section 3.2).

At the onset of this project, the mechanisms by which CCNU, BCNU and NFA impact on GSH homeostasis were not characterized. To administer to this deficiency in the understanding of GSH-dependent toxicity, the overall goal of this project was to investigate the metabolism of nitrosoureas (CCNU and BCNU) and high molecular weight formamides (NFA) as a probe into their toxicity as precursors of isocyanates. With this being the focus, we perceived the following as objectives:

1. To synthesize GSH and NAC conjugates of the potential isocyanates arising in vivo from BCNU (2-chloroethyl isocyanate) and CCNU (cyclohexyl isocyanate, 3-hydroxycyclohexyl isocyanate and 4-hydroxycyclohexyl isocyanate) as reference
compounds. To use the LC/MS/MS properties of these standards to screen for potential metabolites of CCNU and BCNU in the bile and urine of dosed rats, and in urine samples of patients on chemotherapy.

2. To characterize the hepatic microsomal P450 isozyme(s) involved in the metabolism of high molecular weight formamides by using the conversion of N-formyl amphetamine (NFA) to 1-methyl-2-phenylethyl isocyanate (MPIC) as a model system. Microsomal incubations were to be supplemented with GSH to trap MPIC as its thiol conjugate S-[(1-methyl-2-phenylethyl)carbamoyl]glutathione (SMPG), and the latter quantitated by electrospray LC/MS/MS.

3. To characterize the potential involvement of hepatic mitochondrial cytochrome(s) P450 in the bioactivation of formamides to isocyanates. The conversion of NFA to MPIC was to be used as a model. Mitochondrial incubations were to be conducted in the presence of GSH for MPIC to be identified in the form of SMPG by LC/MS/MS.

4. To investigate the release of isocyanates from model thiocarbamates in vitro, and to assess the toxicological impact of this process on model mitochondrial enzyme systems.
II. EXPERIMENTAL

1. Chemicals and materials

1.1. Commercially acquired chemicals and materials

Chemicals and materials were purchased from the following sources:

Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.)
3-Acetamidophenol, AlCl₃, Amberlite® XAD-2 nonionic polymeric adsorbent (20-60 mesh polystyrene), C₆D₆, CDCl₃, cyclohexyl isocyanate, deuterium oxide (99.8 atom % D), dicyclohexylcarbodiimide, digitonin, N-acetyl-L-cysteine, N-hydroxysuccinimide, 4-nitrophenyl chloroformate, rhodium on alumina powder (5 %), trans-4-aminocyclohexanol hydrochloride.

Allied Chemicals (New York, New York, U.S.A.)
Sodium acetate, perchloric acid (70 %, v/v).

Amersham International, plc (Little Chalfont, Buckinghamshire, England)
ECL™ Western blotting detection kit, Hyperfilm™-ECL.

Anachemia Chemicals Ltd. (Montreal, Quebec, Canada)
Ethyl acetate (distilled in glass).

Bachem Inc. (Torrance, California, U.S.A.)
N-t-BOC-S-3,4-dimethylbenzyl-L-cysteine.
BDH Inc. (Vancouver, British Columbia, Canada)

Acetonitrile (glass distilled), di-sodium hydrogen orthophosphate, ethylenediaminetetraacetic acid (EDTA), glycine, magnesium chloride (hexahydrate), methanol (glass distilled), phenobarbitone sodium (phenobarbital), potassium bicarbonate, potassium chloride, potassium hydroxide, silica gel 60 (230 - 400 mesh), sodium chloride, sodium dihydrogen orthophosphate, anhydrous sodium sulfate, solvents, succinic acid, trichloroacetic acid.

Bio-Rad Laboratories (Toronto, Ontario, Canada)

Acrylamide 99.9 %, ammonium persulfate (electrophoresis grade), 2-mercaptoethanol, \( N,N' \)-methylene-bis-acrylamide (BIS), \( N,N,N',N' \)-tetramethylethylenediamine (TEMED), sodium dodecyl sulfate (SDS).

Boehringer Mannheim (Montreal, Quebec, Canada)

Adenosine diphosphate (potassium salt), nicotinamide adenine dinucleotide phosphate-reduced (NADPH), nicotinamide adenine dinucleotide-reduced (NADH), bovine serum albumin (BSA), fatty acid free BSA (fraction V), horse heart cytochrome c (oxidized).

Carnation Inc. (Toronto, Ontario, Canada)

Skim milk powder.

Eastman Kodak Co. (Rochester, New York. U.S.A.)

GBX developer + replenisher, GBX fixer + replenisher.

Fisher Scientific Co. (Fair Lawn, New Jersey, U.S.A.)

Potassium cyanide
Fluka Chemical Corp. (Ronkonkoma, New York, U.S.A.)

4-Aminocyclohexanol (cis/trans 1:2; 50 % in water).

Inter Medico (Markham, Ontario, Canada)

Goat F(ab')\textsubscript{2} anti-rabbit IgG (G+L) horseradish peroxidase conjugate human immunoglobulin adsorbed (TAGO)

J.T.Baker (Phillipsburg, New Jersey, U.S.A.)

BAKERBOND spe\textsuperscript{TM} octadecyl extraction columns (6 mL), silica gel (40 μ particle diameter), sodium dithionite.

Linde Co. (Union Carbide, Vancouver, British Columbia, Canada)

Hydrogen gas.

Mallinckrodt Chemical Works Ltd. (Montreal, Quebec, Canada)

Acetic acid (glacial).

Mandel Scientific Company Ltd. (Guelph, Ontario, Canada)

BA-S 83 supported nitrocellulose transfer and immobilization membrane (pore size 0.2 μm) (Schleicher & Schuell).

Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.)

Potassium carbonate.

Medigas Pacific (Vancouver, British Columbia, Canada)

Carbon monoxide gas (99.5 % purity).
Molecular Probes Inc. (Eugene, Oregon, U.S.A.)

Pentoxysorufin, resorufin.

Pierce Chemical Co. (Rockford, Illinois, U.S.A.)

Heptafluorobutyric acid anhydride, triethylamine (sequanal grade).

Sigma Chemical Co. (St. Louis, Missouri, U.S.A.)

Catalase (2,500 U/mg), 1-chloroethyl-3-cyclohexyl-1-nitrosourea (CCNU), Folin & Ciocalteu's phenol reagent, glutamic acid, glutathione (oxidized), glutathione (reduced), malic acid, metyrapone, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), sucrose, sulfatase (crude solution with β-glucuronidase activity (Type H-2: From Helix pomatia), trifluoroacetic acid (peptide sequencing grade), Trizma base, Trizma HCl, Tween 20, urethane.

Stanchem Ltd. (Winnipeg, Manitoba, Canada)

Ethanol (95%).

Supelco (Oakville, Ontario, Canada)

3 % Dexsil 300 on 100/120 mesh Supelcoport.

Whatman Ltd. (Maidstone, Kent, England)

Silica gel plates (AL SIL G/UV, 250 μm).

1.2. **Compounds and materials which were donated or previously synthesized**

Samples of amphetamine sulfate and N-formyl amphetamine were provided by Dr. R. D. Hossie of the Bureau of Dangerous Drugs, Health Protection Branch (Ottawa, Ontario, Canada).
1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was a gift from Bristol Laboratories of Canada (Belleville, Ontario, Canada).

The primary antibodies, anti-P450 2B1 IgG (19.58 mg-protein/mL) and anti-P450 2C11 IgG (19.95 mg-protein/mL), were donated by the laboratory of Dr. Stelvio Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia, Canada). The antibodies were obtained from the serum of female New Zealand rabbits immunized with electrophoretically pure hepatic microsomal P450 2B1 or P450 2C11 isolated from Long Evans rats (Bandiera and Dworschak, 1992). As determined by immunoblots with available purified P450 isoforms, the antibodies raised to P450 2B1 were polyspecific for P450 2B1, 2B2 and 2B3, and the antibodies raised to P450 2C11 were polyspecific for P450 2C6, 2C7, 2C11 and 2C13.

S-[(1-methyl-2-phenylethyl)carbamoyl]glutathione (SMPG) and N-acetyl-S-[(1-methyl-2-phenylethyl)carbamoyl]cysteine (NMPC) were synthesized in our laboratory by Dr. Abdul Mutlib.

2. Animals, surgical equipment and cell fractionation equipment

Animal Care Facility (University of British Columbia, Vancouver, Canada)

Male Sprague Dawley rats, originally supplied by Charles River (Montreal, Quebec, Canada), were bred locally at the Animal Care Facility. Rats were 275 - 350 g for in vivo xenobiotic metabolism and 160 - 210 g (at the time of sacrifice) for the preparation of subcellular fractions.

Beckman Instruments (Palo Alto, California, U.S.A.)

Model GP centrifuge with GH-3.7 rotor, Model J2-21 centrifuge with JA-20 rotor, Model L8-60M or L5-50 ultracentrifuge with 50.2 Ti rotor.
Becton Dickinson (Rutherford, New Jersey, U.S.A.)

Vacutainer tube 6430, Yale needle 19G 1 1/2"; 23G 1", 25G 1", tuberculosis syringe 1 cc.

Clay Adams (Parsippany, New Jersey, U.S.A.)

Polyethylene tubing PE-10.

Coeval, Inc. (St. Joseph, Illinois, U.S.A.)

Corn cob granules.

Ethicon, Inc. (Somerville, New Jersey, U.S.A.)

000 Silk thread.

Fisher Scientific Co. (Vancouver, British Columbia, Canada)

Micro Centrifuge Model 235 C (13,600 g - fixed).

Hoefer Scientific Instruments (San Francisco, California, U.S.A.)

Model TE50 Transphor Power-Lid and chamber, Model PS500X electrophoresis power supply and chamber.

Purina Mills Inc., (St. Louis, Missouri, U.S.A.)

Purina Laboratory Chow 5001.

Sonics and Materials, Inc. (Dansbury, Connecticut, U.S.A.)

Vibra cell sonicator.

VWR Scientific (London, Ontario, Canada)

Triple Shaft High Torque Stirrer (Talboys).
3. **Instrumentation**

3.1. **Mass spectrometry**

3.1.1. **API LC/MS and LC/MS/MS**

API LC/MS and LC/MS/MS experiments were performed on either a SCIEX API III MS/MS system (Thornhill, Ontario, Canada) or VG QUATTRO (Fisons Instruments, Altrincham, Cheshire, England). Experiments on the SCIEX instrument utilizing heated nebulizer and electrospray interfaces were conducted at Cantest Ltd., Vancouver, British Columbia and The Biomedical Research Centre, University of British Columbia, respectively. APCI (heated nebulizer) experiments were performed by flow injection with the mass spectrometer interfaced to a Waters 600E HPLC. The mobile phase was acetonitrile which was set at a flow rate of 0.2 mL/min. Nebulization was effected with a nebulizer coil temperature of 400 °C and co-axially delivered N2 at a flow rate of 1 L/min. Vapor temperature was estimated at 120°C. APCI generated ions were sampled into Q1, and selected precursor ions were fragmented in the rf-only quadrupole Q2 by collision with argon as the target gas at an energy of 40 eV. Fragment ions were examined in Q3.

Settings for electrospray LC/MS on the SCIEX instrument were ionspray voltage 4,000 V, interface plate voltage 650 V, and orifice lens voltage 50 V. The nebulizer gas was high purity air which was adjusted to a pressure of 40 psi. The instrument was routinely tuned with a mixture of 0.1 mM polypropylene glycols in 2 mM aqueous ammonium acetate/0.1 % formic acid/0.1 % acetonitrile infused at 5 μL/min. Ions scanned were m/z 59.0, 384.3, 520.4, 906.9, 1254.9, 1545.1, 1836.3 and 2010.5. For Q1 scan and LC/MS/MS experiments, dwell times were adjusted to provide a scan rate of 1.85 - 2.15 s/scan. The collision gas was either argon or a mixture of argon:nitrogen 90:10 (v/v). The collision energy was set at 31 eV, and collision gas thicknesses were adjusted to 350 x 10¹² and 480 x 10¹² molecules/cm² for argon and argon:nitrogen, respectively. Chromatography was performed on a Michrom BioResources UMA 600 HPLC fitted with a Reliasil C-18 column (150 x 1 mm, 3 μm particle size). The mobile phase consisted of solvent A: 2 % acetonitrile in 0.05 % trifluoroacetic acid.
(TFA) and solvent B: 80% acetonitrile in 0.05% TFA. Whereas the composition of solvent B was programmed for linear gradients, the composition of solvent A compensated automatically to afford a total of 100% for channels A and B combined. The column flow to the mass spectrometer was subjected to an approximate 1:4 - 1:10 reduction through a flow-split "Tee". Samples were manually injected in a volume of ca 2 - 3 μL, and "blank" injections with solvents B and A were routinely done between HPLC runs to eliminate sample "carryover". HPLC flow rates were 50 μL/min except in LC/MS Methods 6 and 7, in which a linear gradient in flow rate was used. The following are the detailed LC/MS methods.

**LC/MS Method 1.** The mobile phase consisted of 0% B for 2 min, followed by a gradient increase to 50% B at 32 min, a gradient increase to 100% B at 35 min, a hold at 100% B to 47 min, a gradient decrease to 0% B at 48 min, then a hold at 0% B to 60 min. This method was applied to the preliminary screening of NAC conjugates in the urine of patients and rats administered CCNU.

**LC/MS Method 2.** The mobile phase program was 0% B for 6 min, followed by a gradient increase to 100% B at 10 min, a hold at 100% B to 25 min, a gradient decrease to 0% B at 27 min, then a hold at 0% B to 39 min. This method was applied to the SRM analysis of NCEC in the urine of patients on BCNU therapy.

**LC/MS Method 3.** The mobile phase program was a gradient increase from 0% to 70% B at 15 min, followed by a gradient increase to 100% B at 17 min, a hold at 100% B to 29 min, a gradient decrease to 0% B at 31 min, then a hold at 0% B to 43 min. This method was applied to the analysis of conjugated thiol metabolites in the bile of rats dosed with NFA.

**LC/MS Method 4.** The mobile phase program consisted of 0% B for 2 min, followed by a gradient increase to 50% B at 14 min, a gradient increase to 100% B at 17 min, a hold at 100% B to 29 min, a gradient decrease to 0% B at 32 min, then a hold at 0% B to 44 min. This method was applied to the SRM analysis of the conjugated thiol metabolites of NFA in rat bile when compared to synthetic standards; the screening of bile from NCEC-dosed rats; and the quantitation of urinary NAC conjugates of CCNU in rats and humans.
**LC/MS Method 5.** The mobile phase was programmed for 0 % B for 2 min, followed by a gradient increase to 100 % B at 8 min, a hold at 100 %B to 20 min, a gradient decrease to 0 % B at 22 min, then a hold at 0 % B to 32 min. This method was applied to the SRM analysis of SMPG in microsomal incubations.

**LC/MS Method 6.** The mobile phase consisted of 100 % solvent B which was delivered at 20 µL/min for 8 min, followed by a gradient increase in flow to 50 µL/min at 8.5 min, a hold at 50 µL/min to 18.5 min, then a gradient decrease in flow to 20 µL/min at 19 min. This method was applied to the analysis of SMPG in mitochondrial incubations.

**LC/MS Method 7.** In this method both mobile phase and flow rate were modified in gradient fashion. The HPLC program was 0 % B at 50 µL/min for 6 min, followed by a gradient increase to 100 % B and 60 µL/min at 10 min, a hold at 100 % B and 60 µL/min to 25 min, a gradient decrease to 0 % B and 50 µL/min at 27 min, then a hold at 0 % B and 50 µL/min to 39 min. This method was applied to the quantitative analysis of NCEC in the urine of patients on BCNU therapy.

The HPLC flow for direct injection LC/MS/MS experiments on the VG QUATTRO instrument was delivered at 50 µL/min by a Hewlett-Packard 1090II HPLC (Avondale, Pennsylvania, U.S.A.) with a mobile phase of methanol:water (50:50). Samples were injected in volumes of 10 µL. The ionizing voltage was 3,500 V and CAD conditions involved argon as the target gas at an energy of 50 eV and collision cell pressure 1.2 x 10⁻³ Torr.

**3.1.2. Thermospray LC/MS**

Thermospray LC/MS was performed on a Hewlett-Packard System (Avondale, Pennsylvania, U.S.A.) consisting of a Model 1090II HPLC system interfaced to a Model 5989A mass spectrometer. Interface settings were stem temperature 97 °C, filament off, discharge off and fragmenter off. The mobile phase was methanol:ammonium acetate, 5 mM (10:90, v/v) delivered at a flow rate of 1 mL/min.
3.1.3. Capillary column GC/MS

GC/MS of amphetamine was accomplished on a Hewlett-Packard System (Avondale, Pennsylvania, U.S.A.) consisting of a Model 5890 Series II gas chromatograph fitted with a Model 7673 autosampler and interfaced with a capillary split-splitless inlet to a Model 5971A mass selective detector. Samples were injected in a volume of 1 μL and the operation was performed in the splitless mode. Chromatographic separations were effected on a Hewlett-Packard Ultra 2 Crosslinked 5 % phenylmethyl silicone fused silica capillary column (25 m x 0.2 mm i.d. x 0.33 μm film thickness). Column temperature was programmed to increase at 10 °C/min from 100 °C to 170 °C, followed by an increase of 50 °C/min to 250 °C, where it was held for 2 min. Total run time was 18.60 min. Zone temperatures were as follows: injection port 250 °C, transfer line 280 °C, and detector 240 °C. The carrier gas was helium and the column head pressure was maintained at 15 psi. The electron impact mode of ionization was used with an energy of 70 eV and multiplier voltage of 1720 eV. Mass spectrometric acquisition commenced after an 8 min solvent diversion interval. For total ion current (TIC) analyses, the scanning operation was in the linear mode over the range of 40 - 500 a.m.u. TIC mass spectra were recorded as follows. GC/MS: molecular ion m/z (M⁺), other fragment ions, m/z (%). e.g. amphetamine HFBA derivative GC/MS: m/z 331 (M⁺)(0 %), 240 (100 %), 169 (20 %), 118 (90 %), 91 (40 %), 69 (11 %). Selected ion monitoring was performed on the ions m/z 118 (amphetamine), 123 ([²H₃]amphetamine) and 132 (phentermine) with a dwell time of 140 msec/ion.

3.1.4. Packed column GC/MS

Mass spectra of synthetic compounds which were amenable to GC were obtained on a Varian MAT-111 MS interfaced to a Hewlett-Packard 5700A GC fitted with a 1.8 m x 2 mm i.d. glass column packed with 3 % Dexsil 300 on 100/120 mesh Supelcoport. Data were recorded using a Packard Bell computer (IBM AT clone) and a program developed in our laboratory. Mass spectra were recorded from m/z 14 to 750 at 12 scans per minute. Total
ion current (TIC) plots were based on m/z 50 - 500. The electron impact mode of ionization
was used with an energy of 70 eV and emission current of 300 mA. Injection port, line and
separator temperatures were 250°C. The helium carrier gas was set at a flow rate of 25
mL/min. Injections were performed in the splitless mode. Column temperature was
programmed to increase at 32°C/min from 150°C to 300°C, where it was held for 16 min.

3.1.5. Direct probe MS

Direct probe MS was performed on a Nermag R 1010-10C mass spectrometer in the
Department of Chemistry, University of British Columbia. Ion source temperature was held at
200°C. Samples were volatilized by ramping the current across a tungsten filament at 10
mamp/sec. Chemical ionization was induced with NH₃ as the reagent gas.

3.2. NMR Spectroscopy

All NMR spectroscopy was performed in the Department of Chemistry, University of
British Columbia. ¹H-NMR spectra were determined on either a Bruker WH-400 (400 MHz),
Varian XL-300 (300 MHz) or Bruker AC-200 (200 MHz) instrument. ¹³C-NMR spectra
were determined on either a Bruker AMX-500 (125 MHz) or Varian XL-300 (75 MHz).
HMQC spectra were determined on a Bruker AMX-500 with operating parameters ¹³C, 125
MHz and ¹H, 500 MHz. Chemical shifts were measured relative to an external
tetramethylsilane standard. Aromatic ¹³C and ¹H nuclei are designated C' and H', and C" and
H" on monosubstituted and disubstituted phenyl rings, respectively. Peptide-related
methylene protons are distinguished e.g. cysβ, cysβ'. NMR spectra are recorded as follows:
NMR δ (splitting type, coupling constant, integral, assignment). e.g. ¹H-NMR: δ 3.41 (dd, J
= 15 Hz and 5 Hz, 1H, cysβ'). The following terminology is used: s, singlet; bs, broad singlet;
d, doublet; dd, doublet of doublets; q, quartet; m, multiplet; and J, coupling constant in Hz.
3.3. **HPLC**

Quantitative HPLC analyses of the GSH and NAC conjugates (Experimental/Section 5.2.6) were accomplished on a Hewlett-Packard HPLC system consisting of a Model 1090II HPLC fitted with a Spherisorb ODS 5 µm particle size, 250 x 4.0 mm column and Model 3396A integrator. The mobile phase consisted of 10 mM NaH₂PO₄ (pH 4.0):acetonitrile (98.5:1.5, v/v) and was delivered at 1 mL/min.

Purification of metabolites and synthetic compounds (Experimental/Section 5.1.2.2) was performed on a Hewlett-Packard 1050 HPLC connected in sequence to a Beckman Ultrasphere ODS 5 µm particle size, 250 x 4.6 mm column or a Hewlett-Packard Spherisorb ODS 5 µm particle size, 250 x 4.0 mm column, a variable wavelength detector, and a Hewlett-Packard Model 3396A integrator. When necessary, column effluent was delivered to a Gilson FC-80K fraction collector (1 min/fraction). The mobile phase consisted of varying compositions of solvent A: acetonitrile and solvent B: NaH₂PO₄ (10 mM, pH 4.0), and conditions are detailed in Experimental/Section 5.1.2.2. The composition of acetonitrile was programmed, while the composition of the aqueous component compensated automatically to afford a total of 100 % for both channels. Detection wavelength was 210 nm for all HPLC analyses.

3.4. **Ultraviolet/visible Spectrophotometry**

All assays, other than P450 determinations, which involved the use of uv spectrophotometry were performed on a Hewlett-Packard Diode Array 8452A Spectrophotometer and data were recorded on a Hewlett-Packard Vectra Computer with HP 89531A MS-DOS - UV/VIS operating software. Total P450 content was determined on a SLM AMINCO DW-2® double-beam uv-vis spectrophotometer with a MIDAN® II Kinetic Processor/Controller (Urbana, Illinois, U.S.A.). Samples were analyzed in quartz cells of path length 1 cm.
3.5. **Fluorescence spectroscopy**

Fluorescence readings were taken on a Shimadzu (Recording) Spectrofluorophotometer with a RF-540 DR-3 Data Recorder (Kyoto, Japan). For the detection of resorufin, the settings were $\lambda_{\text{excitation}} = 530$ nm, $\lambda_{\text{emission}} = 584$ nm, sensitivity = 1.0 and slit width = 2.0. Reactions were conducted in 2 mL fluorescence cuvettes.

3.6. **Polarography**

Polarographic measurements of oxidative phosphorylation were performed on a Gilson K-IC oxygraph (Middleton, Wisconsin) and an electrode fitted with a standard YSI membrane (Yellowsprings, Ohio).

4. **Methods in chemical synthesis**

4.1. **Chromatography**

Purifications involving medium pressure column chromatography were performed on silica columns of diameter 1.5 or 2.5 cm and variable length depending on the amount of material to be chromatographed (Still et al., 1978). Mobile phases were prepared in order to afford an $R_f$ of 0.3 - 0.5 for the analyte on TLC. If improved resolution was necessary, the polarity of the mobile phase was increased in a gradient fashion during elution. Solvents used as flash chromatography eluants were dried and distilled.

TLC was performed on silica gel plates and compounds detected by either of the following methods. The absorption of u.v. light (aromatics), spraying with a freshly prepared solution of vanillin (1 g) in concentrated $\text{H}_2\text{SO}_4$:ethanol (4:1, v/v, 100 mL) and charring (somewhat selective for thiols), development in $\text{I}_2$ vapour (broad selectivity), spraying with a 0.5 % solution of ninhydrin in ethanol and developing at 60 °C (selective for primary amines).
4.2. Chemical syntheses

*S-(cyclohexylcarbamoyl)glutathione SCCG*

To a saturated solution of GSH (806 mg, 2.62 mmol) in H$_2$O and CH$_3$OH was added cyclohexyl isocyanate (361 µL, 2.83 mmol). The solution was adjusted to pH 8.5 and stirred for 1 h. The reaction was terminated by adjusting to pH 4.0, at which point the product precipitated. The reaction was concentrated to dryness and washed with ether:petroleum ether (1:1) to afford an amorphous white solid (804 mg, 71%). Direct injection LC/MS: m/z 433 (100 %) MH$^+$, 308 (68 %), 290 (10 %), 256 (8 %). LC/MS/MS, Parent ion = m/z 433: m/z 433 (68 %) MH$^+$, 304 (30 %), 233 (12 %), 201 (41 %), 179 (100 %), 162 (52 %), 130 (10 %), 76 (30 %). $^1$H-NMR (CD$_3$OD): $\delta$ 1.12 (m, 4H, H-2$_{ax}$/H-6$_{ax}$, H-3$_{ax}$/H-5$_{ax}$), 1.28 (m, 1H, H-4$_{ax}$), 1.55 (d, 2H, H-3$_{eq}$/H-5$_{eq}$), 1.65 (d, 1H, H-4$_{eq}$), 1.80 (m, 2H, H-2$_{eq}$/H-6$_{eq}$), 1.95 (m, 2H, glub$^\beta$), 2.25-2.40 (m, 2H, gluγγ), 3.10 (m, 1H, cys$^\beta$), 3.30-3.42 (m, 2H, H-i, cys$^\gamma$), 3.65-3.75 (m, glyαα' buried in CH$_3$OH resonance), 4.03 (m, 1H, gluα), 4.48 (q, 1H, cysα).

*N-acetyl-S-(cyclohexylcarbamoyl)cysteine NCCC*

NAC (404 mg, 2.48 mmol) and cyclohexyl isocyanate (350 µL, 2.74 mmol) were dissolved in CH$_3$OH and adjusted to pH 8.5 with stirring. After 1 h the reaction was concentrated to a foam which was washed with ether:petroleum ether (1:1) to afford 762 mg (98 %) of white crystals. Direct injection LC/MS: m/z 289 (100 %) MH$^+$, 306 (15 %) (M+NH$_4$)$^+$, 122 (93 %), 83 (44 %), 76 (21 %), 43 (9 %). $^1$H-NMR (D$_2$O): $\delta$ 1.17 (m, 4H, H-2$_{ax}$/H-6$_{ax}$, H-3$_{ax}$/H-5$_{ax}$), 1.28 (m, 1H, H-4$_{ax}$), 1.56 (d, 2H, H-3$_{eq}$/H-5$_{eq}$), 1.68 (m, 1H, H-4$_{eq}$), 1.80 (m, 2H, H-2$_{eq}$/H-6$_{eq}$), 1.93 (s, 3H, CH$_3$CO), 3.10 (m, 1H, cys$^\beta$), 3.41 (dd, J = 15 Hz and 5 Hz, 1H, cys$^\beta'$), 3.57 (m, 1H, H-1), 4.48 (q, 1H, cysα). $^{13}$C-NMR (D$_2$O): $\delta$ 22.54 (CH$_3$CO), 26.19 (C-4), 26.58 (C-3), 32.04 (cys$^\beta$), 33.87 (C-2), 52.06 (C-1), 54.94 (cysα), 167.65 (SC(=O)NH), 173.11 (CH$_3$CO), 174.12 (COOH).
S-[(4-hydroxycyclohexyl)carbamoyl]glutathione SFHG

4'-Nitrophenyl (trans-4-hydroxycyclohexyl)carbamate. 4-Nitrophenyl chloroformate (1.29 g, 6.40 mmol) was added to a solution of trans-4-aminocyclohexanol (669 mg, 5.82 mmol) in dry distilled CHCl₃. The flask was purged with N₂ and capped with a septum. The reaction was stirred at room temperature for 24 h to afford a yellow supernatant and the product as a white precipitate. The product was filtered, washed with cold CHCl₃ and dried to afford 733 mg (45 %) of white crystals of 4’-nitrophenyl (trans-4-hydroxycyclohexyl)carbamate. TLC, CHCl₃:CH₃OH (25:1), Rf 0.33 (u.v). ¹H-NMR (d₆-DMSO): δ 1.14-1.34 (m, 4H, H₂ax, H₃ax, H₅ax, W₆ax), 1.78-1.88 (m, 4H, H-2, H-3, H-5, H-6), 3.27 (m, 1H, H-1), 3.36 (m, 1H, H-4), 4.50 (d, 1H, -OH), 7.38 (d, J = 12 Hz, 2H, H-2”, H-6”), 7.95 (d, J = 12 Hz, 1H, -NH), 8.25 (d, J = 12 Hz, 2H, H-3”, H-5”). ¹³C-NMR (d₆-DMSO): d 29.10 (C-2), 32.61 (C-3), 48.57 (C-1), 67.06 (C-4), 121.50 (C-2”,C-6”), 124.26 (C-3”,C-5”), 143.18 (C-4”), 151.52 (C-1”), 155.29 (C=O). MS, El, direct probe: m/z 280 (15 %) (M)+, 139 (30 %) (HOC₆H₅NO₂)+, 123 (100 %) (C₆H₅NO₂)+.

A mixture of cis- and trans-isomers of 4’-nitrophenyl (4-hydroxycyclohexyl)carbamate was synthesized by using cis-/trans-4-aminocyclohexanol as the starting material. ¹H-NMR (d₆-DMSO): δ 1.25-1.35 (m, 4H, H-2ax, H-3ax, H-5ax, H-6ax), 1.86-1.96 (m, 4H, H-2eq, H-3eq, H-5eq, H-6eq), 3.40 (m, 1H, H-1), 3.50 (m, 1H, H-4), 6.94 (d, J = 12 Hz, 1H, -NH), 7.27 (d, J = 12 Hz, 2H, H-2”, H-6”), 8.16 (d, J = 12 Hz, 2H, H-3”, H-5”). A less complex pattern was obtained for the resonance at 1.25-1.35 ppm for the cis-/trans-mixture compared to the pure trans-isomer. The occurrence of H-4eq in the cis-isomer experiencing a diminished coupling to H-3ax and H-5ax probably accounts for this phenomenon.

A saturated solution of GSH (42 mg, 0.14 mmol) and the pure trans-isomer of the carbamate (53 mg, 0.19 mmol) in H₂O and CH₃OH was adjusted to pH 8.5, stirred for 1 h, and acidified to pH 5.0. The reaction was concentrated to dryness and washed with ether to afford the product as a white powder in 67 % yield. Direct injection LC/MS: m/z 449 (15 %)
MH⁺, 320 (32 %), 308 (92 %), 290 (100 %), 256 (24 %). LC/MS/MS, Parent ion = m/z 449: m/z 449 (68 %) MH⁺, 320 (27 %), 233 (9 %), 217 (36 %), 179 (100 %), 162 (54 %), 130 (5 %), 76 (27 %). ¹H-NMR (D₂O): δ 1.27-1.37 (m, 4H, H-2ax, H-3ax, H-5ax, H-6ax), 1.92 (bs, 4H, H-2eq, H-3eq, H-5eq, H-6eq), 2.16 (m, 2H, gluββ'), 2.46-2.56 (m, 2H, gluγγ'), 3.18 (m, 1H, cysβ), 3.42 (dd, J = 14 Hz and 4 Hz, 1H, cysβ'), 3.63 (m, 2H, H-1, H-4), 3.70-3.82 (m, 2H, glyα, gluα), 4.63 (m, 1H, cysα). ¹³C-NMR (D₂O): δ 26.46 (gluβ), 29.95 (cysβ), 31.62 (C-2, C-6), 31.80 (gluγ), 43.66 (C-3, C-5), 50.37 (glyα), 53.97 (C-1), 54.42 (cysα), 55.89 (gluα), 69.47 (C-4), 167.90 (S(C=O)NH), 171.70, 174.20, 175.10, 175.30 (GSH carbonyl groups). Assignments at 31.62 and 31.80 ppm may be interchanged.

Using the cis-/trans-mixture of the (4-hydroxycyclohexyl)carbamate as the starting material, a mixture of cis- and trans-isomers of the GSH conjugate was synthesized. The mixture displayed similar LC/MS/MS and ¹H-NMR spectra to the pure trans-isomer and both cis and trans-isomers coeluted under the HPLC conditions used (see Experimental/Section 5.1.2 for HPLC).

*N-acetyl-S-[(4-hydroxycyclohexyl)carbamoyl]cysteine NFHC*

A stirred solution of NAC (38 mg, 0.24 mmol) and 4'-nitrophenyl (trans-4-hydroxycyclohexyl)carbamate (51 mg, 0.18 mmol) in CH₃OH was adjusted to pH 8.5. After 1 h the reaction was terminated by adjusting to pH 5.0, concentrated, and NFHC purified by flash chromatography with CHCl₃:CH₃OH:CH₃COOH (40:5:0.8) to afford 13 mg (24 %) of white crystals. TLC, CHCl₃:CH₃OH:CH₃COOH (10:4:1), Rf 0.33 (H₂SO₄/vanillin). Direct injection LC/MS: m/z 305 (100 %)MH⁺. LC/MS/MS, Parent ion = m/z 305: m/z 305 (68 %)MH⁺, 164 (94 %), 146 (13 %), 122 (100 %), 99 (11 %), 81 (30 %), 76 (15 %), 43(9 %). ¹H-NMR (CD₃OD): δ 1.20-1.45 (m, 4H, H-2ax, H-3ax, H-5ax, H-6ax), 1.87 (m, 4H, H-2eq, H-3eq, H-5eq, H-6eq), 1.97 (s, 3H, CH₃CO), 3.12 (m, 1H, cysβ), 3.41 (dd, J = 12 Hz and 3 Hz, 1H, cysβ'), 3.60 (m, 2H, H-1, H-4), 4.40 (m, 1H, cysα).
A mixture of cis- and trans-isomers of compound NFHC was synthesized by using the cis-trans-mixture of (4-hydroxycyclohexyl)carbamate as the starting material. The mixture displayed similar LC/MS/MS and $^1$H-NMR spectra to the pure trans-isomer and both cis and trans-isomers coeluted under the HPLC conditions used (see Experimental/Section 5.1.2 for HPLC).

$S-$[(3-hydroxycyclohexyl)carbamoyl]glutathione STHG

3-Acetamidocyclohexanol. A stirred suspension of 5 % rhodium on alumina powder (1.0 g) in a solution of 3-acetamidophenol (2.50 g) and acetic acid (1 mL) in ethanol (25 mL) was hydrogenated in a Parr apparatus at 60 °C and 60 psi for 12 h. The reaction mixture was filtered, the residue washed thoroughly with hot CH$_3$OH and the filtrate concentrated to an oil. The oil was taken up into CHCl$_3$, washed with NaHCO$_3$, dried (MgSO$_4$) and purified by flash chromatography (ethyl acetate:CH$_3$OH, v/v, 15:1) to afford 1.46 g (56 %) of 3-acetamidocyclohexanol as off white flakes. TLC, ethyl acetate:CH$_3$OH (10:1), R$_f$ 0.55 (H$_2$SO$_4$/vanillin). GC/MS: m/z 157 (3 %)(M)$^+$, 139 (15 %), 97 (26 %), 72 (52 %), 60 (100 %), 43 (97 %). $^1$H-NMR (CDCl$_3$): δ 1.05-2.10 (m, 8H, H-2, H-4, H-5, H-6), 1.90 (s, 3H, -CH$_3$), 3.10 (s, 1H, -OH), 3.74 (m, 1H, H-3), 4.05 (m, 1H, H-1), 5.85 (d, J = 8 Hz, 0.5H, -NH), 6.27 (d, J = 8 Hz, 0.5H, -NH). The appearance of the amide protons at 5.85 and 6.27 ppm may be due to the presence of rotameric isomers.

3-Aminocyclohexanol. 3-Acetamidocyclohexanol (700 mg, 4.46 mmol) was heated in 3M NaOH under reflux for 20 h. The reaction was concentrated in vacuo to dryness and the product extracted from the residue with ethyl acetate. The extracts were concentrated in vacuo to afford a white paste (441 mg, 86 %). GC/MS: m/z 115(5 %)(M)$^+$, 98(20 %), 72(100 %), 56(50 %), 43(70 %), 30(42 %).

4'-Nitrophenyl (3-hydroxycyclohexyl)carbamate. This carbamate isomer was synthesized from compound 3-aminocyclohexanol according to the procedure described previously for 4'-nitrophenyl (4-hydroxycyclohexyl)carbamate. TLC, CHCl$_3$:CH$_3$OH (25:1),
Rf 0.43 (u.v). 1H-NMR (CDCl3): δ 1.20-2.20 (m, 8H, H-2, H-4, H-5, H-6), 3.60-4.20 (m, 2H, H-1, H-3), 5.10 (d, 0.5H, -NH), 5.80 (m, 0.5H, -NH), 7.30 (d, J = 12 Hz, 2H, H-2", H-6"), 8.25 (d, J = 12 Hz, 2H, H-3", H-5").

The GSH conjugates STHG were synthesized as a mixture from the (3-hydroxycyclohexyl)carbamate isomer according to the procedure detailed for SFHG. LC/MS/MS and 1H-NMR were determined on the mixture. Direct injection LC/MS: m/z 449 (12 %)MH+, 320 (10 %), 308 (100 %), 290 (19 %). LC/MS/MS, Parent ion = m/z 449: m/z 449 (98 %)MH+, 320 (26 %), 308 (12 %), 233 (10 %), 217 (30 %), 179 (100 %), 162 (44 %), 130 (7 %), 76 (17 %). 1H-NMR (D20): δ 1.25-1.35 (m, H-2, H-4), 1.40-1.60 (m, H-6), 1.65-1.74 (m, H-4, H-5, H-6), 1.74-1.88 (m, H-5, H-6), 1.88-2.04 (H-4, H-5, H-6), 2.07 (m, H-4), 2.32 (m, gluββ'), 2.68 (m, gluγγ), 3.35 (m, cysβ), 3.58 (dd, cysβ'), 3.82 (m, H-1, H-3), 3.88-4.00 (m, glyα, gluα), 4.20 (bs, H-1, H-3), 4.80 (m, cysα). 13C-NMR (D2O): δ 19.08 (C-5), 21.47 (C-5), 26.45 (gluβ), 30.84 (cysβ), 31.30 (C-6, cysβ), 31.80 (C-6, gluγ), 33.49 (C-4), 37.94 (C-4), 40.50 (C-2), 43.66 (glyα), 47.36 (C-1), 49.61 (C-1), 53.95 (cysα), 54.39 (gluα), 66.78 (C-3), 68.89 (C-3), 167.89 (S(C=O)NH), 171.67, 174.17, 175.07, 176.40 (GSH carbonyl groups). 1H and 13C assignments were determined by HMQC spectroscopy and no integrations were taken.

N-acetyl-S-[(3-hydroxycyclohexyl)carbamoyl]cysteine NTHC

The NAC conjugates NTHC were synthesized as a mixture from the (3-hydroxycyclohexyl)carbamate isomer as previously described for NFHC. LC/MS/MS and 1H-NMR were determined on the mixture. TLC, CHCl3:CH3OH:CH3COOH (10:4:1), Rf 0.38 (H2SO4/vanillin). Direct injection LC/MS: m/z 305, MH+. LC/MS/MS, Parent ion = m/z 305: m/z 305 (30 %) MH+, 164 (100 %), 146 (10 %), 142 (12 %), 122 (26 %), 81 (14 %), 76 (8 %), 43 (6 %). 1H-NMR (D2O): δ 1.04-2.17 (m, 8H, H-2, H-4, H-5, H-6), 2.07 (s, 3H, CH3CO), 3.16 (m, 1H, cysβ), 3.43 (dd, J = 12 Hz and 1 Hz, 1H, cysβ'), 3.65 (m, 2H, H-
I,H-3), 4.04 (bs, 2H, H-1, H-3), 4.46 (m, 1H, cysα). Chemical shifts for the protons at 1.04 - 2.17 ppm were accurately assigned using HMQC spectroscopy.

S-[(2-chloroethyl)carbamoyl]glutathione SCEG

To a saturated solution of GSH (94.5 mg, 0.31 mmol) in acetonitrile:H2O was added 2-chloroethyl isocyanate in acetone (66 mg, 0.62 mmol). The reaction was stirred for 45 min, during which time the product was formed as a white precipitate. The precipitate was filtered off, washed with acetone and dried in vacuo to afford 72 mg (55 %) of amorphous white solid. In this form the product does not readily dissolve in water or methanol; however, conversion to the HCl salt improves solubility in both solvents. Direct injection LC/MS: m/z 413 (100%) MH⁺, 377 (28%), 351 (23%), 308 (100%). LC/MS/MS, Parent ion = m/z 413: m/z 413 (100%) MH⁺, 308 (4%), 284 (32%), 233 (4%), 181 (82%), 179 (56%), 162 (38%), 130 (24%), 76 (14%). ¹H-NMR (D₂O): δ 2.10 (q, 2H, gluβ'), 2.45 (m, 2H, gluy'), 3.15 (m, 1H, cysβ), 3.35 (dd, J = 14 Hz and J = 4 Hz, 1H, cysβ'), 3.52 (d, J = 3 Hz, 2H, CH₂CH₂Cl), 3.62 (d, J = 3 Hz, 2H, CH₂CH₂Cl), 3.72 (d, 3H, glyα', glu α), 4.60 (q, 1H, cysα).

N-acetyl-S-[(2-chloroethyl)carbamoyl]cysteine NCEC

NCEC was synthesized by a procedure analogous to that for NCCC. The product was obtained as white crystals in quantitative yield. This product had a tendency to melt to a gum at room temperature but its crystallinity could be restored by redissolving it in a saturated solution of acetone/methanol and concentrating it in vacuo. Direct injection LC/MS: m/z 269 (100%) [³⁵Cl]MH⁺, 271 (38%) [³⁷Cl]MH⁺. LC/MS/MS, Parent ion = m/z 269: m/z 269 (100%) MH⁺, 227 (39%), 164 (37%), 146 (6%), 130 (20%), 122 (93%), 106 (23%), 76 (25%), 63 (8%), 43 (6%). ¹H-NMR (D₂O): δ 2.01 (s, 3H, CH₃CO), 3.17 (m, 1H, cysβ), 3.45 (dd, J = 15 Hz and 5 Hz, 1H, cysβ'), 3.56 (m, 2H, CH₂CH₂Cl), 3.65 (m, 2H, CH₂CH₂Cl), 4.42 (q, 1H, cysα).
S-(methylcarbamoyl)glutathione SMG

SMG was synthesized from GSH and MIC by a procedure analogous to that for SCEG. The product was obtained as an amorphous white solid in 48% yield. Direct injection LC/MS/MS, Parent ion = m/z 365: m/z 365 (100%) MH+, 308 (2%), 236 (58%), 233 (5%), 179 (57%), 162 (32%), 133 (67%), 76 (14%). 1H-NMR (D2O): δ 2.15 (q, 2H, gluβ'), 2.45 (m, 2H, gluγγ), 2.75 (s, 3H, N-CH3), 3.15 (m, 1H, cysβ), 3.35 (dd, J = 14 Hz and J = 3 Hz, 1H, cysβ'), 3.80 (t, 1H, glu α), 3.98 (s, 2H, gly αα'), 4.60 (q, 1H, cysα).

N-(1-methyl-2-phenylethyl)formamide (N-formyl amphetamine, NFA)

An aqueous solution of amphetamine sulfate was converted to its free base form by adjusting to alkaline pH, extracted with ether, dried (Na2SO4) and concentrated in vacuo (≥ 4 mmHg, room temperature) to a yellow oil. The amine (423 mg, 3.1 mmol) was dissolved in 20 mL dry, distilled ethyl formate and the mixture refluxed for 20 h. The solvent was removed in vacuo (≥ 6 mm Hg, room temperature) to afford the product (483 mg, 95%) as a pale yellow oil which solidified to a waxy solid on standing. TLC, 0.05% (v/v) triethylamine in ether, Rf 0.32 (u.v.). GC/MS: m/z 163 (0%)(M)+, 118 (80%), 91 (25%), 72 (100%), 44 (55%). 1H-NMR (CDCl3): δ 1.13 (d, J = 8 Hz, 2H, CH3), 1.25 (d, J = 8 Hz, 1H, CH3), 2.65 - 2.90 (m, 2H, CH2), 3.70 (m, 0.2H, CH2CH), 4.35 (m, 0.8H, CH2CH), 5.60 (bs, 0.7H, NH), 6.00 (bs, 0.2H, NH), 7.10 - 7.30 (m, 5H, H-2' - H-6'), 7.80 (d, J = 10 Hz, 0.2H, CHO), 8.05 (d, J = 2 Hz, 0.8H, CHO). The appearance of the CH3, CH2CH, NH and CHO resonances over two chemical shift regions is probably due to the presence of rotameric isomerism.

Aromatic deuteration of NFA was accomplished by AlCl₃ catalyzed exchange with C₆D₆ (Garnett et al., 1972). NFA (228 mg, 1.40 mmol) was dissolved in C₆D₆ and to the stirred solution was added AlCl₃ (608 mg, 4.6 mmol) followed by D₂O (50 µL, 2.7 mmol). The reaction was refluxed for 24 h then quenched with the addition of 5 mL D₂O. The product was extracted with ether, dried (Na₂SO₄) and concentrated in vacuo to a yellow oil. The oil was purified by flash chromatography. CHCl₃ (100%) removed contaminating compounds, and the product was eluted with 100% ether. The product was concentrated to a pale yellow wax in quantitative yield. TLC, ether (100%), Rf 0.32 (u.v.). GC/MS: m/z 168 (1%) (M)+, 123 (80%), 96 (30%), 72 (100%), 44 (55%). The isotopic purity of the product, based on the GS/MS abundances of the isotopically related fragments m/z 91/96 and m/z 118/123 of [²H₆]NFA/[²H₅]NFA, was ≥96 atom %D. ¹H-NMR (CDCl₃): δ 1.10 (d, J = 8 Hz, 2H, CH₃), 1.30 (d, J = 8 Hz, 1H, CH₃), 2.65 - 2.90 (m, 2H, CH₂), 3.70 (m, 0.2H, CH₂CH), 4.30 (m, 0.8H, CH₂CH), 5.60 (bs, 1H, NH), 7.10 - 7.30 (m, 0.02H, H-2'-H-6'), 7.60 (d, J = 10 Hz, 0.2H, CHO), 8.10 (d, J = 2 Hz, 0.8H, CHO). Isotopic purity was ≥99 atom %D based on ¹H-NMR integration of the aromatic protons (H-2' - H-6'). NMR indicated the presence of rotamers.

S-[(1-methyl-2-pentadeuteriophenylethyl)carbamoyl]glutathione [²H₅]SMPG

1-Methyl-2-pentadeuteriophenylethylamine ([²H₅]amphetamine). To a solution of the formamide [²H₅]NFA (135 mg, 0.80 mmol) dissolved in 4 mL CH₃OH was added 4 mL 1 M HCl. The mixture was refluxed for 24 h after which the reaction was complete as determined by TLC. CH₃OH was removed under vacuum, the reaction basified, and the product extracted with ether. The extract was dried (Na₂SO₄) and concentrated in vacuo (≥4 mm Hg, room temperature) to the product as a pale yellow mobile oil in quantitative
yield. TLC, ether (100 %), Rf 0.10 (u.v., ninhydrin). GC/MS: m/z 140 (1 %)(M)+, 96 (6 %), 44 (100 %).

4'-Nitrophenyl (1-methyl-2-pentadeuteriophenylethyl)carbamate. The free base of [2H5]amphetamine (213 mg, 1.52 mmol) was converted to its carbamate by treatment with p-nitrophenyl chloroformate (432 mg, 2.14 mmol) as previously described. After 24 h the reaction was concentrated to an oil, and purified on a silica column (15 x 1.5 cm) with CHCl3:ethyl acetate (20:1, v/v) to afford 151 mg (33 %) of product as pale yellow flakes. TLC, CHCl3:ethyl acetate (20:1, v/v), Rf 0.44 (u.v.). 1H NMR (CDCl3): δ 1.30 (d, J = 8 Hz, 3H, CH3), 2.90 (t, 2H, CH2), 4.05 (m, 1H, CH), 5.00 (d, J = 8 Hz, 1H, NH), 7.10 - 7.20 (m, 2H, H-2", H-6"), 8.25 (d, 2H, J = 10 Hz, H-3", H-5").

S-carbamoylation of GSH with the deuterated carbamate was accomplished under base catalysis as described above. [2H5]SMPG was obtained as off white crystals in 64 % yield. Isotopic yield, based on the LC/MS abundances of m/z 474/469 for [2H5]/[2H0]MH+, was ≥ 99.3 % atom %D. In preparation for NMR, the product was converted to a salt with HCl in methanol/acetonitrile/water solution, and dried under vacuum. Direct injection LC/MS: m/z 474 (100 %) MH+, 469 (0.61 %). LC/MS/MS, Parent ion = m/z 474: m/z 474 (79 %)MH+, 345 (20 %), 308 (4 %), 242 (20 %), 233 (18 %), 179 (100 %), 162 (42 %), 130 (10 %), 124 (15 %), 96 (10 %), 76 (75 %). 1H-NMR (D2O): δ 1.10 (d, J = 11 Hz, 3H, CH3), 2.05-2.15 (m, 2H, gluβ'), 2.40-2.50 (m, 2H, gluγ'), 2.60 - 2.70 (m, 1H, CH2-CH), 2.85 (dd, J = 15 Hz and 4 Hz, 1H, CH2-CH), 3.05 (m, 1H, cysβ), 3.25 (dd, J = 14 Hz and J = 3 Hz, 1H, cysβ'), 3.30 (s, 1H, OCH3)a, 3.70 (s, 2H, glyα'), 3.90 - 4.00 (m, 2H, gluo, OCH3)a, 4.10 (m, 1H, CH-CH3), 4.45 (m, 1H, cysα), 7.20 - 7.30 (m, C6H5)b. aPartial methylation in preparation for NMR or methanol residue; bresonance too low to be integrated.

S-(1-methyl-2-phenylethylcarbamoyl)cysteine SMPC

4'-Nitrophenyl (1-methyl-2-phenylethyl)carbamate. Amphetamine was obtained by CHCl3 extraction of a basified aqueous solution of the sulfate salt, and dried and concentrated
as described for $[^2H_3]$amphetamine. Amphetamine was treated with 4-nitrophenyl chloroformate, as described for the synthesis of 4'-nitrophenyl (4-hydroxycyclohexyl)carbamate, to afford the product in 40% yield. Unlike the (4-hydroxycyclohexyl)carbamate ester, 4'-nitrophenyl (1-methyl-2-phenylethyl)carbamate, was soluble in CHCl₃, and was purified by flash chromatography with CHCl₃:ethyl acetate (20:1, v/v). TLC, CHCl₃:ethyl acetate (20:1, v/v), Rf 0.44 (u.v. detection). $^1$H NMR (CDCl₃): $\delta$ 1.25 (d, J = 8 Hz, 3H, CH₃), 2.85 (t, 2H, CH₂), 4.05 (m, 1H, CH), 4.95 (d, J = 8 Hz, 1H, NH), 7.10 - 7.40 (m, 7H, H-2'-H-6', H-2", H-6"), 8.25 (d, J = 10 Hz, H-3", H-5").

Cysteine (12.3 mg, 0.10 mmol) and 4'-nitrophenyl (1-methyl-2-phenylethyl)carbamate (0.11 mmol) were dissolved in acetonitrile:water (1:1, v/v), the solution adjusted to pH 8.6, and stirred for 90 min. The reaction was terminated by acidifying to pH 4.0. The acetonitrile was removed under vacuum and the aqueous fraction extracted with ether. The product in the aqueous fraction was applied to a C-18 solid phase extraction cartridge washed with water (3 x 6 mL) and eluted in methanol (5 x 6 mL). Concentration of the methanolic extract to dryness afforded 6 mg (19%) of product as white crystals. Direct injection LC/MS: m/z 321 (15%) (M+K)+, 283 (100%) MH+, 122 (25%). LC/MS/MS, Parent ion = m/z 283: m/z 283 (100%) MH+, 122 (85%), 119 (45%), 105 (8%), 91 (65%), 76 (20%). In its acid form, SMPC could not be obtained in sufficiently concentrated solution in either methanol, water, acetonitrile or DMSO. The sodium salt was obtained for NMR by titrating to pH 8.5 with NaOH solution, concentrating the solution to dryness and redissolving in D₂O. However, it was evident that substantial decomposition had occurred. Although complicated by interference, the following resonance signals were considered characteristic of the product SMPC. $^1$H-NMR (D₂O): $\delta$ 1.09 (d, J = 7 Hz, CH₃), 2.65 (m, CH₂CH), 2.75 (dd, J = 10 Hz and 6 Hz, CH₂CH), 3.00 (m, cysβ), 3.08 (dd, J = 18 Hz and 4 Hz, cysβ'), 3.92 (m, CHCH₃), 4.25 (m, cysα), 7.25 (m, C₆H₅).
\textit{S-[(1-methyl-2-phenylethyl)carbamoyl]cysteinylglycine SMPCG}

\textit{N-t-BOC-S-3,4-dimethylbenzyl-L-cysteinylglycine.} \textit{N-t-BOC-S-3,4-dimethylbenzyl-L-cysteine (338.3 mg, 1.0 mmol) and \textit{N}-hydroxysuccimide (127.8 mg, 1.08 mmol) were dissolved in 3 mL dry dioxane with stirring at 10 °C. Dicyclohexylcarbodiimide (243 mg, 1.17 mmol) was added to the solution at 10 °C, the reaction the allowed to reach room temperature, and stirring continued for another 5 h to afford a white suspension. The suspension was filtered, the filtrate added to an aqueous solution (3 mL) of glycine (90.4 mg, 1.20 mmol) and NaHCO\textsubscript{3} (129 mg, 1.54 mmol), and the reaction mixture stirred for 24 h. The reaction was concentrated under vacuum to remove dioxane, extracted (basic) with ethyl acetate (2 x 10 mL), adjusted to acidic pH with 4.0 M HCl, and the desired product extracted (acidic) with ethyl acetate (3 x 10 mL). The crude product was concentrated, and purified by flash chromatography (ether:acetic acid, v/v, 50:0.1) to afford a pale yellow oil. TLC, ether:acetic acid (10:0.1), \textit{Rf} 0.67 (u.v.) LC/MS: m/z 395 (40 %) (M-H)\textsuperscript{-}, m/z 321 (50 %), (M-glycine)\textsuperscript{+}.}

\textit{Cysteinylglycine.} \textit{N-t-BOC-S-3,4-dimethylbenzyl-L-cysteinylglycine was taken up in anisole and transferred to a teflon tube for HF (l) deprotection. The tube was evacuated and HF, introduced as a gas, was allowed to condense at -10 °C (ice/NaCl) in the vessel for the reaction to proceed. The reaction was stirred for 45 min at -10 °C and a further 2 h at room temperature. HF was removed under vacuum to afford the product as an insoluble waxy solid in anisole. The product was taken up into H\textsubscript{2}O (1 mL), extracted with ether (4 x 3 mL), and concentrated \textit{in vacuo} to afford 118 mg (67 %) of product as a white powder.}

\textit{S-carbamoylation of cysteinylglycine was accomplished as described above to afford the product as a white powder in 25 % yield. Direct injection LC/MS: m/z 378 (55 \%) (M+K)\textsuperscript{+}, 340 (100 \%) (M+H)\textsuperscript{+}, 179 (10 \%), 119 (10 \%). LC/MS/MS, Parent ion = m/z 340: m/z 340 (100 \%) MH\textsuperscript{+}, 179 (45 \%), 162 (28 \%), 119 (25 \%), 91 (20 \%), 76 (75 \%). \textit{\textsuperscript{1}H-NMR (D\textsubscript{2}O):} \delta 1.20 (d, J = 10 Hz, 3H, CH\textsubscript{3}), 1.30 (m, 0.2H)\textsuperscript{a}, 2.65 (m, 1H, CH\textsubscript{2}CH\textsubscript{2}), 2.90 (m, 1H, CH\textsubscript{2}CH\textsubscript{2}), 3.2 - 3.5 (m, 2H, cys β, cysβ'), 4.05 (d, 1H)\textsuperscript{b}, 4.10 (s, 2H, glyαα'), 4.20
(m, 1H, CHCH₃), 4.45 (m, 1 H, cysteine). ¹Resonance characteristic of possible minor MPIC decomposition product. ²Uncharacterized contaminant.

N-([1-methyl-2-phenylethyl]carbamoyl)glutathione NMPG

N,N-dile([1-methyl-2-phenylethyl]carbamoyl)glutathione thioether. To 10 mL of an aqueous solution of GSSG (50 mg, 0.081 mmol) was added 5 mL of a solution of 4′-nitrophenyl (1-methyl-2-phenylethyl)carbamate (119 mg, 0.397 mmol) in acetonitrile. The resulting solution was adjusted to pH 9.0 and maintained (at this pH) for 4.5 h with stirring. At that time the carbamate was completely consumed as determined by TLC (TLC conditions, see above). The reaction was concentrated to dryness under vacuum, the resulting residue washed with ether, taken up in H₂O (2 mL) and applied to an Amberlite XAD-2 column (15 x 2.5 cm) which was prepared beforehand by sequential washing with CH₃OH (50 mL), H₂O (50 mL) and 1 % (v/v) HCl solution. Column purification involved elution with H₂O (120 mL), air-drying and removal of the product with CH₃OH (120 mL). The methanolic eluate was concentrated in vacuo to afford the GSSG conjugate as fine white crystals (71 mg, 94 %). TLC, 1-propanol:H₂O:CH₃COOH (15:2:0.4, v/v/v), Rf 0.38 (u.v. and I₂ vapour). HPLC, Hewlett-Packard Spherisorb ODS 2, 5 μm, 250 x 4 mm column, CH₃CN:NaH₂PO₄ (pH 4.0) 22:78, 1 mL/min, tR 5.4 min. LC/MS: m/z 1870.4 (8 %), 1403.2 (16 %), 933.5 (M-H⁻) (100 %), 468.2 (87 %), 306.2 (56 %).

The N-conjugate of GSH was liberated by cleavage of the thioether linkage of the GSSG conjugate with dithiothreitol. To a methanolic solution of the GSSG conjugate (56 mg, 0.060 mmol) was added dithiothreitol (187 mg, 1.2 mmol), the resulting solution adjusted to pH 7.8 and stirred for 3 h. The reaction was concentrated to an oil in vacuo and extracted with ethyl acetate. The resulting off white solid was taken up into CH₃OH and reconcentrated to the final product as off white crystals (55 mg, 92 %). TLC, 1-propanol:H₂O:CH₃COOH (15:2:0.4, v/v/v) Rf 0.58. HPLC, CH₃CN:phosphate buffer (17:83), tR 7.40 min. LC/MS/MS, Parent ion = m/z 469: m/z 469 (32 %) MH⁺, 308 (56 %),
291 (6 %), 259 (6 %), 233 (41 %), 179 (100 %), 162 (60 %), 76 (74 %). $^1$H-NMR (D$_2$O): $\delta$ 0.95 (d, J = 8 Hz, 3H, CH$_3$), 1.90 - 2.10 (m, 2H, glu$\beta'$), 2.30 (m, 2H, glu$\gamma'$), 2.60 (m, 1H, CH$_2$-CH), 2.90 (m, CH$_2$-CH), 3.30 (d, 2H, cys$\beta'$), 3.60 (s, 2H, gly$\alpha'$), 3.90 (m, 1H, glu$\alpha$), 4.10 (m, 1H, CH-CH$_3$), 4.50 (m, 1H, cys$\alpha$), 7.20 - 7.30 (m, 5H, C$_6$H$_5$).

5. Biological experiments

5.1. In vivo xenobiotic metabolism

5.1.1. Metabolism of nitrosoureas in humans

**CCNU Therapy**

Patients on CCNU therapy were under the supervision of Dr. Meg Knowling of the British Columbia Cancer Control Agency (Vancouver, Canada). Patients were orally administered CCNU at 130 mg/m$^2$ and urine collected for up to 8 h after administration of the drug. A 5-mL aliquot of the urine sample was used for the purification of metabolites for APCI LC/MS analysis. For electrospray LC/MS, a 200 µL aliquot was used. Analytical procedures are detailed below.

**BCNU Therapy**

Patients on BCNU therapy were under the supervision of Dr. Glenn Lesser (Johns Hopkins Oncology Centre, Baltimore, Maryland, U.S.A.). A combination regimen of BCNU/cisplatin was administered as a continuous i.v. infusion of both drugs, each at a dosage of 40 mg/m$^2$/day, for three (3) days. Urine samples were taken around the mid-point of the infusion period (i.e. between day 2 and day 3). Other drugs used in concomitant medication included combinations of carbamazepine, dexamethasone, diphenhydramine, furosemide, ondansetron, phenytoin and warfarin. Dosage combinations are detailed in the Results/Section 3.2.2. A 200 µL aliquot of urine was prepared for electrospray LC/MS analysis as described below.
5.1.2. Metabolism of nitrosoureas and formamides in Sprague Dawley rats

5.1.2.1. Xenobiotic administration and collection of urine and bile

General procedures

For qualitative metabolic profiling, three rats (275 - 350 g) per treatment group were administered the following xenobiotics as solutions in corn oil: CCNU (40 mg/mL), BCNU (40 mg/mL) and CEIC (27 mg/mL). Dissolution of the nitrosoureas was facilitated by ca 30 sec of sonication in a 37 °C water bath. NFA: [2H5]NFA, prepared as a 50:50 (w/w) mixture, was dissolved in DMSO (20 mg/mL). NCEC (60 mg/mL) was prepared in aqueous solution. All xenobiotic treatments were delivered by intraperitoneal injection except NCEC which was administered by injection into the tail vein. For specific dosages see Experimental/Section 5.1.2.2. Analysis was conducted on the pooled biological fluid for all studies with one exception. For quantitative metabolic profiling of CCNU in rats, urine samples from each of four dosed animals were individually analyzed.

Urine samples were collected from rats housed in stainless steel metabolism cages with free access to food and water. Urine collected before xenobiotic treatment served as a control.

Bile was collected from the cannulated bile duct of the rat. Rats were anesthetized by intraperitoneal injection of urethane (1.2 g/kg) which was administered as an aqueous solution (0.4 g/mL). After anesthesia was induced (ca 30 min) an incision of 3 cm was made on the ventral surface of the animal left of the abdominal mid-line toward the diaphragm. The intestines were displaced, the portion of the bile duct at the junction with the duodenum located and surrounding tissue gently removed with cotton wool. A puncture was made into the duodenum with a 25G1 needle to access the bile duct. Through this aperture a bevelled cannula of PE-10 tubing was inserted 2 cm into the bile duct, ligated at the duodenal junction with silk thread, and exteriorized through the lower abdominal wall guided by the bore of an externally inserted 19G1 1/2 needle. The displaced intestines were relocated into the
abdominal cavity and the incision closed with interrupted sutures. Depending on the experiment, bile samples were collected from 4 to 18 h post-dose and pooled for analysis. Where possible, bile collected before xenobiotic administration served as control, otherwise the bile of rats dosed with the solvent vehicle was used for this purpose.

To minimize the likelihood of metabolite decomposition, collected urine and bile samples were removed every 2 to 3 h and stored at -20 °C until preparation for analysis. Prior to analysis, samples were centrifuged (13,600 g, 15 min, Micro Centrifuge) to pellet particulate matter.

5.1.2.2. Metabolism studies

Metabolism of CCNU in rats and humans. Metabolic profiling using HPLC purification and APCI LC/MS.

In the initial studies on CCNU metabolism, rats were dosed i.p. with CCNU (130 mg/kg) and urine or bile collected for 18 h post-dose. Urine and bile samples (5 mL) were adjusted to pH 5.0 with sodium acetate buffer (1.0 M, pH 5.0) and enzymatically deconjugated with sulfatase (200 U)/β-glucuronidase (10,000 U) at 37 °C for 12 h. Samples were extracted with ether, the organic extract discarded, the aqueous fraction concentrated to dryness and then reconstituted in methanol (500 μL). The metabolite NCCC was identified in urine by direct injection LC/MS and LC/MS/MS without further purification, whereas the metabolites NFHC and NTHC were purified by HPLC (Experimental/Section 3.3). Chromatographic conditions for the separation of NFHC and NTHC involved the following conditions. HPLC was programmed at 4 % B for 16 min, followed by a gradient increase to 100 % B at 18 min, a hold at 100 % B to 26 min, a gradient decrease to 4 % B at 28 min, then a hold at 4 % B to 35 min. The flow rate was 0.75 mL/min for 9 min, followed by a gradient increase to 1.0 mL/min at 10 min, then held at 1.0 mL/min to 35 min. Under these conditions the metabolites NFHC and NTHC eluted at 8.2 min and 12.8 - 14.2 min, respectively. HPLC
fraction 8 and fractions 11-15, corresponding to these retention times were concentrated and analyzed by APCI LC/MS and LC/MS/MS.

Urinary metabolic profiling of CCNU in humans was performed on one patient (#91-20848). A 5 mL aliquot of the urine was prepared and analyzed as described above.

For the purification of GSH conjugates from bile, the mobile phase consisted of 2.5 % B for 14 min, followed by a gradient increase to 5 % B at 15 min, a hold at 5 % B to 20 min, a gradient increase to 15 % B at 21 min, a hold at 15 % B to 28 min, a gradient increase to 100 % B at 30 min, then a hold at 100 % B to 39 min, a gradient decrease to 2.5 % B at 41 min, then a hold at 2.5 % B to 46 min. The flow rate was 1.0 mL/min throughout the entire run. Under these conditions the metabolites SFHG, STHG and SCCG eluted at 11.9, 18.8-19.7 and 26.5 min, respectively. HPLC fractions 11-12, 18-20, and 25-26, corresponding to these retention times, were concentrated and analyzed by APCI LC/MS and LC/MS/MS.

Metabolism of CCNU in rats and humans. Quantitative urinary metabolite profiling with electrospray LC/MS/MS.

Rats (n = 4) were dosed i.p. with CCNU (50 mg/kg), and urine collected for 24 h post-dose. Two 200 μL aliquots of urine from each rat and from each of two patients on chemotherapy (#91-20848 and #90-24692) were analyzed for CCNU metabolites.

Preliminary screening for the metabolites NFHC, NTHC and NCCC was performed using LC/MS Method 1. For quantitative analyses, standard curves for the urinary metabolites NFHC, NTHC and NCCC, were prepared from stock solutions of the synthetic standards in 0.5 % TFA (v/v) of 464.5, 972.5 and 523.5 nmol/mL, respectively. The internal standard was NCEC 2,450 nmol/mL in 0.5 % TFA (v/v). Dilutions of the stock solutions into a final volume of 200 μL with distilled water were used to prepare standards of the following concentrations: 11.63, 23.25, 46.50, 116.25 and 139.50 nmol/mL for NFHC; 24.33, 48.65, 97.30, 243.25 and 291.90 nmol/mL for NTHC; and 13.10, 26.20, 52.40, 131.00, and 157.20 nmol/mL for NCCC. Aliquots (200 μL) of urine samples and standard solutions were mixed
with 50 µL (122.5 nmol) of internal standard, centrifuged and the supernatant analyzed by LC/MS Method 4 with SRM for the transitions m/z 305/164 (NFHC and NTHC), 289/164 (NCCC) and 269/164 (NCEC, internal standard).

**Metabolism of BCNU and 2-chloroethyl isocyanate in rats.** *Metabolite profiling using HPLC purification and APCI LC/MS.*

Groups of rats were dosed with BCNU (130 mg/kg) or CEIC (30 mg/kg) from which urine or bile were collected for 18 h post-dose. Urine and bile samples (5 mL) were extracted with ether, the organic extract discarded, the aqueous fraction concentrated to dryness and then reconstituted in methanol (500 µL). Metabolites were purified by HPLC using an isocratic delivery of 1.5 % B at 1 mL/min (Experimental/Section 3.3). Under these conditions SCEG and NCEC eluted at 6.8 min and 9.4 min, respectively.

**Metabolism of BCNU in humans.** *Quantitative urinary metabolite profiling in patients with electrospray LC/MS/MS*

Preliminary screening for the metabolite NCEC was performed using LC/MS Method 2. For quantitative analyses, the standard curve for the metabolite NCEC was prepared from a stock solution of the synthetic standard in 0.5 % TFA (v/v) of 70.8 nmol/mL. The internal standard was NCCC 250 nmol/mL in 0.5 % TFA (v/v). Appropriate volumes of stock solution, spiked with 75 µL (18.8 nmol) internal standard, and diluted to a final volume of 300 µL with distilled water were used to prepare standards of the following concentrations: 2.36, 4.72, 9.44, 11.80 and 17.70 nmol/mL.

Aliquots (200 µL) of urine from five patients on BCNU therapy were analyzed for NCEC in duplicate. The urine aliquot was diluted with 25 µL distilled water spiked with 75 µL internal standard, centrifuged and the supernatant analyzed by LC/MS Method 7 with SRM for the transitions m/z 269/122 (NCEC) and 289/164 (NCCC, internal standard).
Metabolism of NCEC in rats. Quantitative biliary metabolite profiling with electrospray LC/MS/MS.

NCEC was administered at 140 mg/kg by tail vein, and bile collected for 12 h. A 90 μL aliquot of bile was mixed with 10 μL 1.0 % TFA (v/v), the mixture centrifuged and the supernatant analyzed by LC/MS Method 4 with SRM for the transitions m/z 413/384, 413/181, 413/179 (SCEG); and 269/164, 269/122 (NCEC).

Metabolism of NFA in rats. Metabolite profiling with electrospray LC/MS/MS.

NFA:[2H5]NFA was administered at 20 mg/kg and urine collected for 24 h post-dose. Subsequent to urine collection, rats were anesthetized and their bile ducts cannulated. Immediately after cannulation, animals were dosed for a second time as described above and bile collected for 4 h over sodium acetate buffer (1.0 M, pH 5.0).

Bile and urine samples (2 mL) were extracted with ethyl acetate (4 x 3 mL) and the aqueous fractions applied to a column of XAD-2 resin (7 x 1.5 cm). The column was washed with water (30 mL), and dried with air. The metabolite containing fraction was eluted with methanol (50 mL), concentrated to dryness, and reconstituted in 500 μL of methanol for analysis. Bile and urine samples were all analyzed by LC/MS Method 3, except where biliary and synthetic samples were compared using LC/MS Method 4. Transitions are detailed in Results/Section 4.

5.2. In vitro xenobiotic metabolism

5.2.1. Maintenance and treatment of rats

Upon arrival rats (twelve per treatment group) were allowed one day to acclimatize and were maintained on 12-h light cycles with free access to water and Purina 5001 rat chow. Treatments were phenobarbital (PB) and acetone, with untreated rats serving as controls. Rats were intraperitoneally administered sodium phenobarbital prepared as an aqueous solution (75 mg/mL) at 75 mg/kg for five days (Shayiq and Avadhani, 1990). Using an
adaptation of a previously reported method (Hyland et al. 1991), acetone prepared as a 50 % aqueous solution (v/v) was administered at 5 mL/kg for two days by oral gavage. Rats were killed 24 h after the conclusion of treatment, and at that time weighed 180 - 210 g.

5.2.2. Preparation of mitochondria, mitoplasts and microsomes

Hepatic subcellular fractions were prepared by adaptations of the methods of Shayiq et al., (1991) for mitochondria and mitoplasts and Thomas et al. (1983) for microsomes. Rats were stunned with a blow to the head and decapitated. In brisk sequence, an incision was made along the abdominal midline, the liver removed, and immersed in ice-cold Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, final concentration). All subsequent steps were performed on ice. Livers were washed free of blood, then transferred to a plastic beaker with isolation buffer (2 mM HEPES, 70 mM sucrose, 220 mM D-mannitol, 2 mM EDTA, pH 7.5, final concentration) containing 1 mM dithiothreitol and 0.5 mg/mL fatty acid free BSA where they were minced with a scissors, and again washed free of blood. Each minced liver was placed into approximately 30 mL isolation buffer/dithiothreitol/BSA for homogenization (3-4 passes with setting 10 of stirrer). Note: Mitochondria are fragile organelles and high homogenization rates should be avoided.

Isolation of cellular fractions

Cellular fractions were isolated by differential centrifugation (4 °C) of the liver homogenate. The supernatant resulting from two centrifugations at 1,000 g for 10 min was retained and the pellet (nuclear debris and intact cells) discarded. The postnuclear supernatant was centrifuged at 12,000 g for 15 min to afford a mitochondrial pellet and microsomal/ cytosolic supernatant. Mitochondria were washed three times (12,000 g, 10 min) in isolation buffer and either resuspended in 0.25 M sucrose (ca 1/2 pellet volume) and used as such or further purified. Mitochondria were purified by treatment with digitonin to remove the outer mitochondrial membrane and associated microsomal adherence (Shayiq et al., 1991).
Removal of the mitochondrial outer membrane to produce mitoplasts was accomplished with two treatment cycles with a freshly prepared digitonin solution. Digitonin used for this purpose was crystallized twice from ethanol, and prepared as a 2% solution by dissolution in hot isolation buffer then stored on ice. Mitochondria were adjusted to a concentration of 40-60 mg/mL in isolation buffer as determined by differential absorbance at 280 and 310 nm (Clarke, 1976) and stirred for 15 min with digitonin added at 1.8 μL/mg-protein. The resulting mitoplasts were diluted 10-fold in isolation buffer and sedimented (12,000 g for 10 min). Mitoplasts were washed five times with isolation buffer, resuspended in 0.25 M sucrose, sonicated and stored at -70°C.

Microsomes were isolated as a pellet from the postmitochondrial supernatant by centrifugation at 105,000 g for 70 min, washed with a solution of KCl (1.15%, w/v) in EDTA (10 mM, pH 7.4), resuspended in 0.25 M sucrose (ca 3 x pellet volume) and stored at -70°C (Thomas et al., 1983). (See Guengerich (1982) for a comprehensive review of microsome preparation and analysis.)

**Analysis of cellular fractions**

The integrity of mitochondria required for metabolism experiments was evaluated by the performance of oxidative phosphorylation and the release of citrate synthase activity upon sonication (Experimental/Section 5.2.4).

Protein concentrations of cellular fractions were determined in duplicate with a modified Lowry procedure (Markwell et al., 1978). A standard curve of protein concentrations was generated using BSA.

Total cytochrome P450 content was determined by measuring the difference spectrum of the dithionite reduced P450-CO complex (λ = 450 nm, ε = 91 cm⁻¹ mM⁻¹) (Omura and Sato, 1964). P450 analyses in microsomes and mitoplasts were conducted in triplicate on a SLM AMINCO DW-2® double-beam spectrophotometer.
5.2.3. *In vitro* metabolism of NFA

5.2.3.1. Metabolism of NFA by microsomes

*Preparation of the P450 inhibitors*

In incubations where the influence of inhibitors was examined, either of 2.5 mM orphenadrine-HCl, 50 mM sodium diethyldithiocarbamate (DEDTC) or 50 mM metyrapone was added in an appropriate volume to afford a final incubation concentration of 0.025, 0.5 and 1.0 mM, respectively. Orphenadrine and DEDTC were prepared in aqueous solution and adjusted to pH 7.6, and metyrapone was dissolved in methanol. In incubations serving as control for the inhibitors, water or methanol was substituted as necessary.

*Incubation procedure for NFA microsomal metabolism*

Duplicate microsomal incubations were conducted using the following method. A mixture containing 500 µL of 200 mM KH₂PO₄ (pH 7.4), 100 µL of 60 mM GSH (pH 7.4), 10 µL of 300 mM MgCl₂, 10 µL of 100 mM NADPH, 10 µL of 100 mM NADH, and 1.0 nmol of microsomal P450 was preincubated in a total volume of 990 µL at 37 °C for 10 min. The reaction was initiated by the addition of 10 µL of 500 mM methanolic NFA, allowed to proceed for 5, 10, 20 or 30 min, and terminated by the addition of 100 µL of 20 % (v/v) TFA. The formation of SMPG (see below for analysis) was found to be linear up to 10 min. Subsequent experiments were conducted for a 10 min reaction time, following a 10 min preincubation in the presence of either of the following agents: 10 µL of DEDTC, 10 µL of orphenadrine, 20 µL of metyrapone, or 10 µL catalase (prepared as a solution of 100 U/µL in 200 mM KH₂PO₄, pH 7.4). (See above for the preparation of inhibitor solutions). For experiments involving the use of denatured microsomal protein, microsomes prepared from untreated rats were immersed in boiling water for 4 min.
**LC/MS analysis of SMPG**

The standard curve used to determine the concentration of SMPG in microsomal incubations was prepared from a stock solution of 91.74 nmol/mL of SMPG standard and 113.0 nmol/mL of \(^{2}\text{H}_5\)SMPG internal standard in water:methanol (80:20, v/v). Standard solutions, prepared by mixing an appropriate volume of SMPG stock solution and 500 μL of 200 mM KH\(_2\)PO\(_4\) (pH 7.4) in a final volume of 1.0 mL (with water), generated concentrations of 1.84, 4.60, 9.20, 18.40 and 36.80 nmol/mL. Each of the standards (as in the case of the microsomal incubations) was subsequently treated with 100 μL of 20% TFA (v/v). An aliquot of 180 μL from the standard solutions and microsomal incubations was removed, spiked with 20 μL of \(^{2}\text{H}_5\)SMPG solution (2.3 nmol), centrifuged, and analyzed by LC/MS Method 5 with SRM for the transitions m/z 469/340 (SMPG) and 474/345 (\(^{2}\text{H}_5\)SMPG).

**GC/MS analysis of amphetamine**

Amphetamine sulfate was prepared in an aqueous stock solution to afford an amphetamine concentration of 92.32 nmol/mL. Standard dilutions of concentration 0.46, 2.30, 4.60, 9.20, 18.40, 27.60 and 36.80 nmol/mL in a volume of 1.0 mL with KH\(_2\)PO\(_4\) (pH 7.4) buffer were treated with 100 μL of 20% TFA (v/v). An aliquot of 180 μL from each of the standard solutions and microsomal incubations was transferred to another test tube, spiked with 20 μL of \(^{2}\text{H}_5\)SMPG solution (2.3 nmol) and 100 μL of 82.90 nmol/mL phentermine HCl (8.3 nmol). This mixture was basified with 2 mL of 0.5 M K\(_2\)CO\(_3\)/KHCO\(_3\) (pH 10.5) and extracted with 5 mL of hexane:ether:triethylamine:isopropanol (400:100:5:5, v/v/v/v). The organic extract was concentrated under vacuum in a Savant AS290 automatic SpeedVac Concentrator (Savant Instruments Inc., Farmingdale, New York) to a pressure of 1000 μm Hg. The concentrate was taken up into 250 μL of 0.00125 M triethylamine in toluene and derivatized with 20 μL heptafluorobutyric acid anhydride (HFBA) at 60 °C for 1 h. The derivative solution was backwashed with 5 mL of 0.1 M phosphate buffer (pH 7.4) by mixing for 5 min, and a 150 μL aliquot of the organic supernatant was transferred to borosilicate
microvial inserts for analysis by GC/MS (Experimental/Section 3.1.3). SIM was performed for the ions m/z 118 (amphetamine), 123 ([2H₅]amphetamine) and 132 (phentermine).

5.2.3.2. Metabolism of NFA by intact mitochondria

Duplicate mitochondrial incubations were conducted as follows. A mixture containing 500 μL of incubation buffer (8 mM HEPES, 6 mM KH₂PO₄, 2 mM EDTA and 500 mM sucrose, pH 7.4, final concentration), 100 μL of 60 mM GSH (pH 7.4), 50 μL of a mixture of glutamate/malate (168 mM/66 mM, pH 7.4) and ca 13 mg of intact well-coupled mitochondria (used immediately upon isolation) was preincubated in a total volume of 990 μL at 30 °C for 10 min. The reaction was initiated by the addition of 10 μL of 500 mM methanolic NFA, allowed to proceed for 90 min, and terminated by the addition of 200 μL of 200 mM HCl. A 200 μL aliquot of the incubation was diluted with 200 μL of 200 mM HCl, extracted with ether (2 x 600 μL) and the aqueous fraction applied to a C-18 extraction cartridge. The cartridge was washed with water (3 x 2 mL), dried, and the desired product eluted with methanol (3 x 2 mL). The methanolic extracts were concentrated to dryness, reconstituted in 20 μL methanol and analyzed by SRM using LC/MS Method 6. The analyte was monitored with the transitions m/z 469/340 and m/z 469/237.

5.2.3.3. Metabolism of NFA by sonicated mitoplasts

The incubation procedure for NFA with sonicated mitoplasts and the method for the analysis of SMPG in mitoplast incubations were as described above for microsomes with the following exceptions. The incubation duration was 90 min (instead of 10 min). The amount of mitochondrial P450 used per incubation varied from 0.30 nmol - 1.00 nmol of spectrally determined P450, depending on the available mitoplast protein from the preparation.
5.2.4. Enzyme assays

5.2.4.1. Citrate synthase assay for mitochondrial/mitoplast intactness

The assay for citrate synthase was performed according to the procedure described by Shepherd and Garland (1969). This enzyme which functioned as a marker for the mitochondrial matrix activity was marginally detectable in intact mitochondria (or mitoplasts), but was completely uncovered upon sonication (2 x 20 sec-pulse/20 sec-wait cycles on ice) and present in the supernatant upon centrifugation (13,600 g, 15 min, Micro Centrifuge). The principle of the assay resided upon the free thiol of coenzyme A (CoA), released from acetyl coenzyme A upon conversion of oxaloacetic acid (OAA) to citric acid, initiating cleavage of the disulfide linkage of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to afford the thiolate anion (Scheme 8). Progress of the reaction was followed by the increase of absorbance at 412 nm. The reaction was performed in 0.1 M Tris-HCl buffer (pH 8.0), and solutions of DTNB, acetyl CoA and OAA were prepared in the same buffer and adjusted to pH 8.0 if necessary. Into 1.0 mL of 0.1 M Tris-HCl buffer were added 20 μL of 10 mM DTNB (0.2 μmol), 20 μL of 5 mM acetyl CoA (0.1 μmol) and 20 - 50 μL of the supernatant of intact or sonicated mitochondrial or mitoplast protein (ca 4 mg/mL) to afford a total volume of 1190 μL. After recording the level of non-specific activity as the baseline, 10 μL of 50 mM OAA (0.5 mmol) was added to initiate the reaction. The net rate of reaction before and after the addition of OAA was attributed to the activity of citrate synthase. The level of mitochondrial or mitoplast integrity was calculated as follows:

\[
\text{Percent integrity} = \frac{\text{Activity of enzyme (sonicated)} - \text{Activity of enzyme (intact)}}{\text{Activity of enzyme (sonicated)}} \times 100
\]
Scheme 8. Principle of citrate synthase assay showing the enzyme catalyzed conversion of acetyl CoA to CoA coupled to the cleavage of DTNB to afford the thiolate which absorbs at 412 nm.

5.2.4.2. Glutathione reductase

Glutathione reductase (GR) activity was measured by following the decrease in absorbance at 340 nm as NADPH is consumed in the conversion of GSSG to GSH (Carlberg and Mannervik, 1985). Minor modifications to this method involved using the phosphate buffer at pH 7.6 as opposed to pH 7.0, ambient temperature instead of 30 °C, and initiating the reaction with GSSG and not the enzyme. GR present in the mitochondrial matrix was released upon sonication and analyzed in the supernatant upon centrifugation (13,600 g, 15 min, Micro Centrifuge). A typical assay proceeded as follows. To 500 μL of a buffer containing KH₂PO₄ and 2 mM EDTA (pH 7.6, final concentration) were added 200 - 300 μL of the supernatant of sonicated mitochondria (ca 4 mg/mL), and water to afford 1 mL. The contents of the cuvette were adjusted to zero absorbance, 50 μL of 2 mM NADPH (prepared
as a solution in 10 mM Tris.HCl, pH 7.6) added and the rate of decrease in absorbance measured to obtain a baseline level of non-specific activity. The reaction was initiated with the addition of 100 µL of 20 mM GSSG (pH 7.6) and the rate again measured. The net rate before and after the addition of GSSG was ascribed to the activity of GR.

5.2.4.3. NADPH-cytochrome c reductase

The assay for KCN-insensitive NADPH-cytochrome c reductase, a marker for microsomal activity, was based on the NADPH-catalyzed formation of reduced cytochrome c which absorbs at 550 nm. The procedure reported by Lake (1987) was used with the following modifications: the incubation volume was halved, the reaction was performed at room temperature (and not 37 °C), and a Diode array spectrophotometer was used (thereby circumventing experimental provisions for a dual beam instrument). Into a cuvette were added 500 µL of 100 mM K2HPO4 (pH 7.6), 100 µL of 15 mM KCN, 0.2 mg protein solution (microsomes, mitochondria or mitoplasts) and 500 µL of 0.125 mM oxidized cytochrome c to afford a volume of 1.2 mL. After recording the level of non-specific activity as a baseline, 50 µL of 10 mM NADPH was added to initiate the reaction. The net rate (recorded as nmol/min/mg-protein) derived from the difference in rates before and after the addition of NADPH was considered to be due to microsomal NADPH-cytochrome c reductase activity. The microsomal contamination in mitoplasts (or mitochondria) was based on (1) specific activity and (2) the recovery in mitoplasts of the microsomal marker from the liver homogenate. The latter method of evaluating purity was used by Niranjan et al. (1985).

\[
\text{microsomal contamination (\%)} = \frac{\text{specific activity of mitoplasts}}{\text{specific activity of microsomes}} \times 100
\]

\[
\text{microsomal contamination (\%)} = \frac{\text{specific activity of mitoplasts}}{\text{specific activity of liver homogenate}} \times \frac{\text{yield of mitoplast protein}}{\text{yield of liver homogenate protein}} \times 100
\]
5.2.4.4. Oxidative phosphorylation

Into the stirred polarograph cell were placed 1 mL of isolation buffer (see Experimental/Section 5.2.2), 3 - 5 mg mitochondrial protein, 20 µL of 500 mM phosphate buffer (pH 7.5) and 80 µL of substrate. The substrate (adjusted to pH 7.5) was either 82.5 mM succinic acid or a mixture with a final concentration of 168 mM glutamic acid and 66 mM malic acid. After the addition of substrate, state IV oxidative phosphorylation was recorded until O\textsubscript{2} consumption stabilized. To initiate state III oxidation 10 - 25 µL of 14 mM ADP (pH 7.5) was added. After the state III oxidative burst had transpired and state IV respiration was restored, another aliquot of ADP was added to generate another cycle. Repeat experiments (n = 3 - 5) were performed until the O\textsubscript{2} in the cell was exhausted. The following parameters were calculated to evaluate mitochondrial integrity (Estabrook, 1967; Heisler, 1991).

\[
\text{respiratory control ratio (RCR)} = \frac{\text{rate of state III respiration}}{\text{rate of state IV respiration}}
\]

\[
\text{ADP/O ratio} = \frac{[\text{ADP}]\text{added}}{[\text{O}_2]\text{consumed}}
\]

5.2.4.5. p-Nitrophenyl hydroxylase

Microsomal p-nitrophenyl hydroxylase activity, a selective biotransformation for P450 2E1, was quantitated through the formation of the product 4-nitrocatechol which absorbs light at 546 nm in alkaline solution (Scheme 9) (Reinke and Moyer, 1985; Koop, 1986). A mixture of 500 µL of K\textsubscript{2}HPO\textsubscript{4} (pH 6.8), 100 µL of 1 mM NADPH, and 2.0 nmol of spectrally determined microsomal P450 was preincubated in a total volume of 0.9 mL at 30 °C for 10 min either with or without inhibitor (10 µL) (Experimental/Section 5.2.3.1). The reaction was initiated by the addition of 100 µL of 0.1 mM p-nitrophenol, allowed to proceed for 10 min,
and terminated with the addition of 500 μL of 0.6 M HClO₄. The reaction mixture was centrifuged (920 g, 5 min, Beckman Model GP), 1 mL of the supernatant removed, and basified with 100 μL of 10 M KOH. A white crystalline by-product which was formed was sedimented by another centrifugation (920 g, 5 min) and the supernatant analyzed spectrophotometrically. The concentration of the microsomal product was determined from a curve of standard concentrations of authentic 4-nitrocatechol, prepared in 1 mL volumes and treated with HClO₄ and KOH in an analogous fashion to the incubations. Activities were recorded as nmol 4-nitrocatechol/min/nmol-P450.

![Scheme 9. Hydroxylation of p-nitrophenol to 4-nitrocatechol, the anion of which is detected by the absorption of light at 546 nm.](image)

5.2.4.6. Pentoxyresorufin-O-dealkylase

The dealkylation of pentoxyresorufin to resorufin, demonstrated by Burke et al. (1985) to be a sensitive and selective assay for P450 2B1/2B2, was used as the probe for these isoforms in microsomal preparations (Scheme 10). Into 1.93 mL of buffer containing 100 mM HEPES, 5 mM MgCl₂ and 0.1 mM EDTA, were added 10 μL of 50 mM NADPH (prepared as a solution in the HEPES buffer), 30 μL of 4 mg/mL microsomal protein and 20 μL water or 20 μL inhibitor (see Experimental/Section 5.2.3.1). Microsomes were allowed to preincubate at 37 °C for 5 min, after which the reaction was initiated by the addition of 10 μL of a 1 mM solution of pentoxyresorufin in dimethylsulfoxide. The progress of the biotransformation was monitored by recording the increase in the fluorescence of resorufin every 30 sec for 7 min. Excitation and emission wavelengths for resorufin were 530 nm and
584 nm, respectively. The concentration of resorufin in microsomal incubations was determined from a standard curve of authentic resorufin. Activities were recorded as nmol resorufin/min/mg-protein.

![Scheme 10](image.png)

Scheme 10. Monitoring of PROD activity by the formation of the fluorescent product resorufin.

5.2.5. Electrophoretic separation and immunoblotting of proteins

Separation of mitoplast and microsomal proteins was accomplished by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) (Laemmli, 1970). The separating gel containing in final concentration 0.375 M Tris-HCl (pH 8.8), 0.10 % (w/v) SDS, 7.5 % (w/v) acrylamide-bis, 0.042 % (w/v) ammonium persulfate and 0.03 % (v/v) $N,N,N',N'$-tetramethylethylenediamine (TEMED) and stacking gel containing 0.125 M (pH 6.8) Tris-HCl, 0.10 % (w/v) SDS, 3.0 % (w/v) acrylamide-bis, 0.08 % (w/v) ammonium persulfate and 0.05 % (w/v) TEMED were sequentially cast so as to afford 3.5 cm of stacking gel above 14 cm of separating gel with standard thickness 0.75 mm. Proteins were prepared as solutions of concentration 0.1 - 1 mg/mL for mitoplasts and 0.002 - 0.2 mg/mL for microsomes in a buffer which contained in final concentration 0.031 M (pH 6.8) Tris-HCl, 0.5 % (w/v) SDS, 0.0005 % (w/v) bromophenol blue, 5 % (v/v) glycerol and 2.5 % (v/v) 2-mercaptoethanol. Samples were immersed in boiling water for 2 min, cooled on ice, and introduced on to the gels in 20 μL volumes which afforded loadings of 2 - 4 μg-mitoplast protein and 0.04 - 4 μg-microsomal protein per well. Proteins were electrophoretically separated on the gel by applying a constant current across a buffered bath of 0.1 M Trizma base, 0.767 M glycine, 0.4 % w/v SDS (final
concentration) at 10 °C. The current was maintained at 10 - 12 mA/gel for ca 1 h followed by 20 - 25 mA/gel for ca 2 h which allowed for migration of the dye front through the stacking gel and separating gel, respectively.

Immediately following egress of the dye front from the separating gel, electrophoretic separation was discontinued, the stacking gel was discarded and the separating gel was mounted in a Hoefer TE 50 Transphor® unit for electrophoretic transfer of the resolved proteins on to a nitrocellulose membrane (Towbin et al., 1979). Transfer was effected at 4 °C by maintaining a constant current of 0.4 A for 2 h across a bath solution with a final concentration of 20 % (v/v) methanol, 0.02 M Tris, 0.154 M glycine and 0.008 % (w/v) SDS.

After transfer, the nitrocellulose membrane was immunochemically probed. The membrane was subjected to sequential treatments with 50 mL-solutions (vide infra) in a Nalgene™ box which allowed for the complete immersion of the blot when laid horizontally. Treatments were conducted in an incubating water bath at 37 °C. Solutions which were used in this procedure were termed as follows: phosphate buffered saline (137 mM NaCl, 2.6 mM KCl, 8.1 mM Na₂HPO₄, 1.5 mM KH₂PO₄ and 0.2 mM EDTA, final concentration in distilled water), blocking buffer (1 % (w/v) fatty acid free BSA and 3 % (w/v) skim milk powder, final concentration in phosphate buffered saline), antibody dilution buffer (0.05 % Tween 20, final concentration in blocking buffer), and wash buffer (0.05 % (v/v) Tween 20 in phosphate buffered saline). The membrane with the transferred protein was first treated with blocking buffer at 4 °C overnight to eliminate non-specific protein binding and then washed with wash buffer (3 x 10 min, 37 °C).

Development of the membrane for the detection of specific protein binding involved the treatment sequence: primary antibody exposure → wash cycle (wash buffer, 3 x 10 min, 37 °C) → secondary antibody exposure → wash cycle (wash buffer, 5 x 10 min, 37 °C). The primary antibodies were anti-P450 2B1 IgG and anti-P450 2C11 IgG, prepared at final concentrations of 2.0 μg/mL and 40 μg/mL, respectively, in antibody dilution buffer. The
secondary antibody was horseradish peroxidase conjugated goat \textit{anti}-rabbit IgG, prepared as a 1:3000 (v/v) dilution in antibody dilution buffer.

Immunologically recognized proteins were detected by the enzymatic activity of the conjugated secondary antibody using ECL™ Western Blotting methodology. The principle of this technique involves the peroxidase/H$_2$O$_2$-catalyzed generation of the chemiluminescent excited state of the compound luminol which is adsorbed to the surface of the blot. The blot was wrapped in SaranWrap™ for photographic analysis. The location of the bands on the blot, which could be visualized by their luminescence in a darkroom, facilitated the introduction of the photographic film. The film (Hyperfilm™-ECL) was briskly brought into contact from above, pressed against the blot in a cassette, and briskly removed. Exposure times of \textit{ca} 5 sec were generally adequate for a suitable photographic impression. Such was the sensitivity of this technique, that \textit{strict observation of the above procedure was necessary to avoid multiple exposures and blurring of bands on the photographic imprint}. After exposure, the film was sequentially immersed with gentle shaking in Kodak GBX developer for \textit{ca} 30 sec, in Kodak GBX fixer for 1 min and in water for 2 min.

The intensity of the bands generated on film was measured by computer image analysis with a VISAGE 110 Bio Image Analyzer (Bio Image, Ann Arbor, Michigan) consisting of a high resolution camera and a Sun Microsystems Workstation using software options of Whole Band Analysis and Integrated Intensity. Microsomal contamination in mitoplasts was calculated using immunodetectable P450 2B2 and P450 2C11 as illustrated below.

\[
\text{microsomal contamination (\%) } = \frac{\text{Integrated intensity of mitoplasts}}{\text{Loading of mitoplasts}} \times \frac{\text{Loading of microsomes}}{\text{Integrated intensity of microsomes}} \times 100
\]
5.2.6. Evaluation of the reactivity and toxicity of thiocarbamate conjugates

5.2.6.1. Decomposition of thiocarbamate conjugates

*Incubation procedure for thiocarbamate decomposition*

The following compounds were prepared as stock solutions for standard curves and reaction incubations: 5 mM SCEG and 5 mM NCEC in distilled water; 12.5 mM GSH and 12.5 mM NAC in 10 mM NaH$_2$PO$_4$, and adjusted to pH 7.4; and 0.185 mM NFHC in 10 mM NaH$_2$PO$_4$ and adjusted to pH 2.0.

In experiments performed in triplicate, 0.4 mL of 5 mM SCEG was added to 1.6 mL of 12.5 mM NAC to afford final concentrations of 1.0 mM and 10.0 mM, respectively. At intervals of 0.3, 0.5, 1.0, 1.3, 2.3 and 3.0 h aliquots of 100 μL were withdrawn and added to 400 μL of the internal standard NFHC for HPLC analysis (*vide infra*).

The reaction of 1 mM NCEC and 10 mM GSH (final concentrations) was conducted in an experiment of analogous design, but instead the reaction was probed at 0.5, 1.08, 1.5, 2.0, 3.0, 4.0 and 5.0 h. The decomposition of SCEG (final concentration 1.0 mM) in 10 mM NaH$_2$PO$_4$ was also investigated with duplicate determinations made at 0.3, 1.1, 3.4, 4.4 and 6.4 h.

*HPLC analysis of reaction mixtures*

Appropriate volumes of SCEG and NCEC stock solution, 10 mM NaH$_2$PO$_4$ buffer (pH 4.0) and 400 μL of internal standard to afford a total volume of 500 mL were used to generate standard concentrations of 0.02, 0.05, 0.10, 0.20 and 0.30 mM NCEC (final concentration). Samples were analyzed on a Hewlett-Packard 1090II HPLC (see Experimental/Section 3.3.).

5.2.6.2. Inhibitory action of SCEG toward mitochondrial glutathione reductase

Freshly prepared mitochondria (14 mg) in 2.4 mL isolation buffer (for composition see Experimental/Section 5.2.2.) were preincubated for 10 min at 30 °C. To this suspension was
added 600 μL of 5 mM SCEG (solution prepared in isolation buffer and adjusted to pH 7.6) or 600 μL isolation buffer (control). The final concentration of SCEG in the incubation was 1 mM. At 7 min, 1.05 h, 2.05 h, 4.05 h and 6.25 h were removed aliquots of 450 μL of which 150 μL and 300 μL were designated for citrate synthase (Experimental/Section 5.2.4.1.) and GR (Experimental/Section 5.2.4.2.) assays, respectively. In preparation for the GR assay, mitochondria were diluted to 1 mL with isolation buffer, centrifuged (13,600 g, 10 min, Micro Centrifuge), washed with isolation buffer, and resuspended in the original 300 μL volume. Recorded values are the mean % change of separate test experiments (n = 3) compared to control experiments (n = 2).

5.2.6.3. Inhibitory action of SMG toward oxidative phosphorylation

Freshly prepared mitochondria (8 mg) in 750 μL isolation buffer (see Experimental/Section 5.2.2.) were preincubated at 30 °C for 10 min. To this suspension was added 50 μL of 50 mM SMG (solution prepared in isolation buffer and adjusted to pH 7.6) or 50 μL of isolation buffer (control), and the incubation maintained for 90 min. At that time, a 400 μL aliquot was removed for analysis of oxidative phosphorylation (Experimental Section 5.2.4.4). Tests of oxidative phosphorylation were performed in triplicate, and recorded values are the mean of two separate test and control experiments.

6. Statistical analysis

The Student's one way, two sample t-test was used to compare paired groups of data. A level of statistical significance was attributed to values of p < 0.05.
III. RESULTS

1. Synthetic Chemistry

1.1. Synthesis of thiocarbamate conjugates

The synthesis of thiocarbamate conjugates was approached by using nucleophilic thiolate addition to the isocyanate (Scheme 11), or nucleophilic thiolate substitution of 4-nitrophenoxide from the 4-nitrophenyl carbamate ester (Scheme 12). Nucleophilic addition of the cysteiny1 thiols of GSH and NAC to cyclohexyl isocyanate and 2-chloroethyl isocyanate in methanol/water or acetonitrile/water proceeded rapidly to afford the thiocarbamate esters in high yield. Using a procedure developed in our laboratory (Mutlib et al., 1991), 4-nitrophenyl carbamoyl esters were synthesized by treatment of 4-hydroxycyclohexylamine, 3-hydroxycyclohexylamine, amphetamine or [2H5]amphetamine with 1.1 equivalent of 4-nitrophenyl chloroformate. This stoichiometry of reagents allowed selective carbamoylation of the primary amine over the hydroxyl group in the case of 4- and 3-aminocyclohexanol (Scheme 12). Trans-4-aminocyclohexanol was commercially available either as the pure trans-isomer or a mixture of cis- and trans-isomers. Thus, (4-hydroxycyclohexyl)carbamoyl conjugates of GSH and NAC were synthesized as either the pure trans- or a mixture of cis- and trans-isomers. 3-Aminocyclohexanol was synthesized by catalytic hydrogenation of the phenyl ring of 3-acetamidophenol followed by base hydrolysis of the amide (Hilton and Walker, 1975b) (Scheme 13). The resulting 3-aminocyclohexanols occurred as a mixture of two pairs of enantiomers inasmuch as two chiral sites were generated at the substituted 1- and 3-positions of the cyclohexyl ring. In turn, conversion of the 3-aminocyclohexanols to carbamoylated derivatives of GSH and NAC incorporated additional chiral sites of fixed stereochemistry through the thiol moiety. Consequently, a mixture of four diastereomers ensued for (3-hydroxycyclohexyl)carbamoyl conjugates of GSH and NAC.

HPLC analyses for synthetic thiol conjugates were developed for application to the isolation of CCNU and BCNU metabolites from urine and bile. One such method for the
separation of synthetic (hydroxycyclohexyl)carbamoyl conjugates of NAC is shown in Figure 7. *Trans-* and *cis-*isomers of NFHC coeluted at 8.2 min, whereas the four diastereomers of NTHC were well-resolved at 12.4, 12.9, 13.9 and 15.1 min.

Scheme 11. Synthesis of thiocarbamate conjugates, typified in the case of SCCG, by nucleophilic addition of the GSH thiolate to cyclohexyl isocyanate.

Scheme 12. Synthesis of thiocarbamate conjugates, typified in the case of SFHG, by nucleophilic substitution of the 4-nitrophenoxide anion by the thiolate of GSH.
Scheme 13. Synthesis of 3-(hydroxycyclohexyl)carbamoyl derivatives of GSH and NAC. Positions indicated with an asterix are synthetically generated chiral centres. Conversion of the primary amine to the thiocarbamate is detailed in Scheme 12.

Figure 7. HPLC analysis of (hydroxycyclohexyl)carbamoyl derivatives of NAC. Conditions: Hewlett-Packard Spherisorb ODS 5 μm particle size, 250 x 4.0 mm column, acetonitrile:10 mM NaH₂PO₄ (pH 4.0) (4:96), 1 mL/min. *cis*- and *trans*-isomers of NFHC coeluted at 8.2 min and the diastereomers of NTHC eluted at 12.4 - 15.1 min.
In addition to GSH and NAC, other thiols, namely cysteine and cysteinylglycine were also employed as nucleophiles for substitution reaction with the 4-nitrophenylcarbamoyl ester of amphetamine. Thus, SMPG, NMPC, SMPC and SMPCG were synthesized as reference compounds for MPIC-derived metabolites of NFA (Scheme 14).

1.2. Synthesis of aromatically deuterated compounds

The pentadeuteriophenylderivatives of amphetamine that were synthesized included $[^2\text{H}_5]\text{NFA}$ for the administration to rats and $[^2\text{H}_5]\text{SMPG}$, required as an internal standard in the analysis of SMPG. $[^2\text{H}_5]\text{Amphetamine}$ was synthesized from amphetamine (95 % yield overall) through protection of the amine by $N$-formylation followed by Fiedel-Crafts catalyzed deuterium exchange with C$_6$D$_6$ and finally $N$-deprotection with aqueous methanolic acid (Scheme 15). The rationale for this approach to the deuterated amine is noteworthy. Attempts to introduce deuterium into the phenyl ring of the amine without $N$-formyl protection resulted in a mixture of incompletely labelled isomers (as judged by GC/MS) regardless of the duration, temperature or the stoichiometry of AlCl$_3$ and C$_6$D$_6$. This result, in light of the facile deuterium incorporation into the formamide (GC/MS isotopic purity $\geq 96$ atom %D), is considered due to inactivation of the AlCl$_3$ catalyst by the primary amine.
(Garnett et al., 1972). Deuterium was retained throughout the intervening synthetic steps: 
$[^{2}\text{H}_5]\text{amphetamine} \rightarrow[^{2}\text{H}_5]\text{carbamate ester} \rightarrow[^{2}\text{H}_5]\text{SMPG}$ to afford the product in $\geq 99\%$ isotopic purity based on the LC/MS abundances of m/z 474/469 for $[^{2}\text{H}_5]/[^{2}\text{H}_0]\text{MH}^+$.

Scheme 15. Synthesis of $[^{2}\text{H}_5]\text{NFA}$ and $[^{2}\text{H}_5]\text{SMPG}$ from amphetamine. Conversion of the primary amine to the thiocarbamate is detailed in Scheme 12.

1.3. Synthesis of N-carbamoylated GSH

$N\{}[(\text{1-methyl-2-phenylethylcarbamoyl})\text{glutathione} \text{ (NMPG)} \text{ was synthesized as a reference compound for a putative metabolite of NFA. Because treatment of GSH with the} 4'$-nitrophenyl (1-methyl-2-phenylethyl)carbamate resulted in preferential carbamoylation of the thiol over the primary amine, an alternative strategy for the synthesis of NMPG was sought whereby the thiol could be selectively protected. This possibility was presented in the use of oxidized glutathione (GSSG) as the starting material. Treatment of GSSG with the carbamate ester afforded the NMPG thioether which was cleaved under mild conditions with dithiothreitol to liberate the thiol function of NMPG (Scheme 16).
Scheme 16. Synthesis of $N$-[(1-methyl-2-phenylethylcarbamoyl)]glutathione (NMPG). The initial step involves reaction of the glutamyl primary amine of oxidized glutathione with the 4-nitrophenyl carbamate ester of amphetamine.

2. Analysis of thiocarbamate conjugates

Thiol conjugates were analyzed by mass spectrometry and NMR spectroscopy, with the former being the principal analytical technique. API LC/MS was accomplished with either a heated nebulizer or ionspray interface. LC/MS/MS was effected by CAD with argon or an argon:nitrogen mixture. Minimal use was made of thermospray LC/MS and the results provided by this technique are addressed briefly.

2.1. Atmospheric pressure ionization mass spectrometry of thiol conjugates

In an attempt to systematize the fragmentation nomenclature of the conjugates of GSH and its derivatives, it was necessary to incorporate descriptions of fragment formation along the peptide chain and across the thiol linkage. Description of the fragmentation of GSH and NAC conjugates along the peptide backbone is based on the nomenclature of Roepstorff and Fohlman (1984). Cleavage sites designated A, B and C occur with charge retention at the $N$-
terminal, and cleavage sites designated X, Y and Z occur with charge retention at the C-terminal (Tables 3 - 5, Figure 8). In order to address the issue of thiol associated cleavages, the nomenclature of Deterding et al., (1989) is employed. According to this convention, fragmentation is partitioned on either side of the S-linkage. \( R_G \) denotes GSH, cysteine or NAC-derived fragments, \( R_X \) represents the xenobiotic-derived moiety, and proton transfers are not detailed. All thiol conjugates in this report are to be considered S-linked, except in the case of NMPG which is N-linked and is distinguished as such. The description of fragmentations on either side of the N-linkage for NMPG is analogous to the convention for S-conjugates.

Introduction of GSH conjugates by heated nebulizer to APCI LC/MS resulted in considerable thermally induced fragmentation (Table 3). Two discernable fragmentation pathways for GSH conjugates involved either conservation or loss of the the substrate-distinguishing \( R_X \) fragment. In the first case, loss of the glutamyl moiety ensued with proton transfer to \( Y_2 \). In the second case, loss of \( R_X \) with proton transfer to the glutathionyl moiety \( R_{GS} \) initiated a cascade involving the sequential loss of \( H_2O \) and \( H_2S \) to afford the fragment ions \( m/z \) 290 and \( m/z \) 256, respectively. The extent of fragmentation promoted by the heated nebulizer varied with the GSH conjugate (Table 3). Whereas \( MH^+ \) of SCCG at \( m/z \) 433 dominated its APCI \( Q_1 \) mass spectrum, with less carriage of the ion current by the GSH-derived fragments, \( MH^+ \) of SFHG and STHG at \( m/z \) 449 registered a diminished intensity (\( \leq 25 \% \)), accompanied by a prominent appearance of \( Y_2, R_{GS} (GSH), R_{GS-H_2O} \) and \( R_{GS-H_2O-H_2S} \) fragments. Of the GSH conjugates analyzed, only SCEG underwent APCI fragmentations within the substrate moiety incurring losses of \( HCl \) and \( CH_2CH_2Cl \) to afford the ions \( m/z \) 377 and \( m/z \) 351, respectively.

CAD of carbamoylated GSH conjugates, as illustrated for SFHG in Figure 8, revealed common fragmentations within this class of compounds (Table 5). Only two fragment ions, \( Y_2 \) and \( A_Y Y_2 \), carried information about the thiocarbamoyl linkage, and these were generally of moderate intensity. By and large, the thiol related fragment ions at \( m/z \) 179
(cysteinylglycine, R\textsubscript{G}SY\textsubscript{2}), 162 (R\textsubscript{G}SZ\textsubscript{2}) and 76 (glycine, Y\textsubscript{1}) carried the major portion of the ion current. Fragment ions related solely to the substrate did not contribute significantly to the LC/MS/MS of car bamoylated GSH conjugates.

NMPG, like S-carbamoylated GSH conjugates, revealed a propensity to fragment under CAD with prominent fragment ions at m/z 179 and 162; however, the tendency toward fragmentation with ions at m/z 130 (R\textsubscript{G}NB\textsubscript{1}) and 308 (GSH, R\textsubscript{G}N) was more pronounced with NMPG. Furthermore, NMPG was clearly distinguished from its regioisomer SMPG by the absence of S-carbamoyl associated ions at m/z 340 (Y\textsubscript{2}) and 237 (A\textsubscript{2}Y\textsubscript{2}) (Figure 9).

APCI LC/MS of car bamoylated NAC conjugates resulted in little apparent fragmentation in the mass range scanned for \textit{Q\textsubscript{1}}, \textit{viz} m/z 200 - m/z 400. CAD of the NAC conjugates, typified in the case of NFHC in Figure 10, afforded diagnostic daughter ions for the thiol moiety at m/z 164 (NAC, R\textsubscript{G}S) and 122 (cysteine, R\textsubscript{G}SY\textsubscript{1}), with minor fragmentation directed toward the substrate part of the molecule (Table 5).

2.2. Thermospray mass spectrometry of thiol conjugates

Thermospray LC/MS spectra were obtained for native GSH and the car bamoylated NAC conjugate NFHC. Although LC/MS operation was performed with the filament, discharge and fragmenter all set in the "off" mode to optimize pseudomolecular ion intensity, substantial fragmentation was still observed for both compounds. The pseudomolecular ion of GSH at m/z 308 occurred as the base peak accompanied by fragment ions m/z 129, 147, and 179 (Figure 11). Ascribed to C\textsubscript{1}-H\textsubscript{2}O, C\textsubscript{1} and Y\textsubscript{2}, respectively (Bean et al., 1990), these ions are also characteristic of GSH conjugates (Parker et al., 1988; Rashed et al., 1989). The abundance of the proposed fragment ions in the thermospray LC/MS spectrum of NFHC decreased in the order R\textsubscript{X}S-H\textsubscript{2}O (m/z 159) > R\textsubscript{G}S (m/z 164), R\textsubscript{G} (m/z 130) with little carriage of the ion current ascribed to the pseudomolecular ion (m/z 305, 3 %) (Figure 12).
Table 3. Characteristic $Q_1$ fragment ions of GSH conjugates under APCI LC/MS.

<table>
<thead>
<tr>
<th>GSH conjugate</th>
<th>$\text{MH}^+$ (%)</th>
<th>$\text{Y}_2$ (%)</th>
<th>$\text{RGS}$ (%)</th>
<th>$\text{RGS-H}_2\text{O}$ (%)</th>
<th>$\text{RGS-H}_2\text{O-H}_2\text{S}$ (%)</th>
<th>Other diagnostic fragment ions (%)</th>
<th>100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCG</td>
<td>433 (100)</td>
<td>304 (3)</td>
<td>308 (68)</td>
<td>290 (10)</td>
<td>256 (8)</td>
<td>377 (28)</td>
<td>433</td>
</tr>
<tr>
<td>SFHG</td>
<td>449 (15)</td>
<td>320 (32)</td>
<td>308 (92)</td>
<td>290 (100)</td>
<td>256 (24)</td>
<td>351 (23)</td>
<td>290</td>
</tr>
<tr>
<td>STHG</td>
<td>449 (12)</td>
<td>320 (10)</td>
<td>308 (100)</td>
<td>290 (19)</td>
<td>256 (6)</td>
<td></td>
<td>308</td>
</tr>
<tr>
<td>SCEG</td>
<td>413 (60)</td>
<td>284a</td>
<td>308 (100)</td>
<td>290a</td>
<td>256a</td>
<td></td>
<td>308</td>
</tr>
</tbody>
</table>

$a$ Below scanned mass range.
Table 4. Characteristic fragment ions of carbamoylated GSH conjugates under API LC/MS/MS.

<table>
<thead>
<tr>
<th>GSH conjugate</th>
<th>MH⁺ (%)</th>
<th>B₁ (%)</th>
<th>Y₁ (%)</th>
<th>Y₂ (%)</th>
<th>A₂Y₂ (%)</th>
<th>RG⁻S (%)</th>
<th>RGSB₂ (%)</th>
<th>RO⁻SY₂ (%)</th>
<th>RGS⁻Z₂ (%)</th>
<th>RX related fragments (%)</th>
<th>100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCCG</td>
<td>433 (68)</td>
<td>130 (10)</td>
<td>76 (30)</td>
<td>304 (30)</td>
<td>201 (41)</td>
<td>308 (6)</td>
<td>233 (12)</td>
<td>179 (100)</td>
<td>162 (54)</td>
<td>83 (6)</td>
<td>179</td>
</tr>
<tr>
<td>SFHG</td>
<td>449 (68)</td>
<td>130 (5)</td>
<td>76 (27)</td>
<td>320 (27)</td>
<td>217 (36)</td>
<td>308 (6)</td>
<td>233 (9)</td>
<td>179 (100)</td>
<td>162 (54)</td>
<td>81 (4)</td>
<td>179</td>
</tr>
<tr>
<td>STHG</td>
<td>449 (98)</td>
<td>130 (7)</td>
<td>76 (17)</td>
<td>320 (26)</td>
<td>217 (30)</td>
<td>308 (12)</td>
<td>233 (10)</td>
<td>179 (100)</td>
<td>162 (44)</td>
<td>81 (4)</td>
<td>179</td>
</tr>
<tr>
<td>SCEG</td>
<td>413 (100)</td>
<td>130 (24)</td>
<td>76 (14)</td>
<td>284 (32)</td>
<td>181 (82)</td>
<td>308 (4)</td>
<td>233 (4)</td>
<td>179 (56)</td>
<td>162 (38)</td>
<td>413</td>
<td></td>
</tr>
<tr>
<td>SMG</td>
<td>365 (100)</td>
<td>130 (0)</td>
<td>76 (14)</td>
<td>236 (58)</td>
<td>133 (67)</td>
<td>308 (2)</td>
<td>233 (5)</td>
<td>179 (57)</td>
<td>162 (32)</td>
<td>365</td>
<td></td>
</tr>
<tr>
<td>SMPG</td>
<td>469 (81)</td>
<td>130 (6)</td>
<td>76 (81)</td>
<td>340 (20)</td>
<td>237 (16)</td>
<td>308 (4)</td>
<td>233 (20)</td>
<td>179 (100)</td>
<td>162 (51)</td>
<td>119 (14) 91 (12)</td>
<td>179</td>
</tr>
<tr>
<td>²[H]₅SMPG</td>
<td>474 (79)</td>
<td>130 (10)</td>
<td>76 (75)</td>
<td>345 (20)</td>
<td>242 (20)</td>
<td>308 (4)</td>
<td>233 (18)</td>
<td>179 (100)</td>
<td>162 (42)</td>
<td>124 (15) 96 (10)</td>
<td>179</td>
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</tbody>
</table>
Table 5. Characteristic fragment ions of NAC conjugates under API LC/MS/MS.

<table>
<thead>
<tr>
<th>NAC conjugate</th>
<th>MH⁺ (%)</th>
<th>B₁ (%)</th>
<th>Y₁ (%)</th>
<th>Rₖ (%)</th>
<th>RₖS (%)</th>
<th>RₖSY₁ (%)</th>
<th>RₖS-H₂O (%)</th>
<th>RₖY₁-CH₂ (%)</th>
<th>RX related fragments (%)</th>
<th>100 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCC</td>
<td>289 (56)</td>
<td>43 (9)</td>
<td>247 (0)</td>
<td>130 (5)</td>
<td>164 (100)</td>
<td>122 (93)</td>
<td>146 (7)</td>
<td>76 (21)</td>
<td>83 (44)</td>
<td>164</td>
</tr>
<tr>
<td>NFHC</td>
<td>305 (68)</td>
<td>43 (9)</td>
<td>263 (4)</td>
<td>130 (7)</td>
<td>164 (94)</td>
<td>122 (100)</td>
<td>146 (13)</td>
<td>76 (15)</td>
<td>99 (11)</td>
<td>81 (30)</td>
</tr>
<tr>
<td>NTHC</td>
<td>305 (30)</td>
<td>43 (6)</td>
<td>263 (4)</td>
<td>130 (6)</td>
<td>164 (100)</td>
<td>122 (26)</td>
<td>146 (10)</td>
<td>76 (8)</td>
<td>99 (12)</td>
<td>81 (14)</td>
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<td>NCEC</td>
<td>269 (100)</td>
<td>43 (6)</td>
<td>227 (39)</td>
<td>130 (20)</td>
<td>164 (37)</td>
<td>122 (93)</td>
<td>146 (6)</td>
<td>76 (25)</td>
<td>106 (23)</td>
<td>63 (8)</td>
</tr>
<tr>
<td>NMPC</td>
<td>325 (31)</td>
<td>43 (0)</td>
<td>283 (0)</td>
<td>130 (4)</td>
<td>164 (87)</td>
<td>122 (100)</td>
<td>146 (8)</td>
<td>76 (7)</td>
<td>91 (5)</td>
<td>119 (20)</td>
</tr>
</tbody>
</table>

---

![Chemical Structures](image)
Figure 8. API LC/MS fragment ion spectrum of S-[(4-hydroxycyclohexyl)carbamoyl]glutathione (SFHG) obtained by CAD of MH⁺ at m/z 449 illustrating the fragmentation system used in this report.
Figure 9. API LC/MS fragment ion spectra of (A) N-[(1-methyl-2-phenylethyl)carbamoyl]glutathione (NMPG) and (B) S-[(1-methyl-2-phenylethyl)carbamoyl]glutathione (SMPG) obtained by CAD of MH⁺ at m/z 469.
Figure 10. API LC/MS fragment ion spectrum of N-acetyl-S-[(4-hydroxcyclohexyl)carbamoyl]cysteine (NFHC) obtained upon CAD of MH+ at m/z 305.
Figure 11. Thermospray LC/MS of glutathione with MH\(^+\) at m/z 308.

Figure 12. Thermospray LC/MS of N-acetyl-S-[(4-hydroxycyclohexyl)carbamoyl]cysteine (NFHC) with MH\(^+\) at m/z 305.
2.3. **NMR spectroscopy of thiol conjugates**

Mass spectral information was complemented by NMR spectral data. Conversion of cysteiny1 thiols to thiocarbamates resulted in resolution of the non-equivalent methylene protons and a downfield move of the chemical shift of the α-proton from ca 4.03 ppm to ca 4.40 to 4.80 ppm (Figure 13). The substrate component of thiol conjugates sometimes gave rise to complex spectra which were resolved by two-dimensional NMR techniques. The cyclohexylcarbamoyl moiety of SCCG and NCCC gave rise to an involved resonance pattern for protons in the chemical shift range 1.12 ppm to 1.80 ppm. Proton assignments for the cyclohexyl ring, as well as the thiol moiety were deciphered using $^1$H-$^1$H correlated spectroscopy (COSY) as typified in the case of SCCG (Figure 14). Consistent with protons in cyclic systems (Silverstein *et al.*, 1981), the resonance of the unsubstituted axial protons at 1.12 (H-2$_{ax}$/H-6$_{ax}$, H-3$_{ax}$/H-5$_{ax}$) and 1.28 (H-4$_{ax}$) ppm appeared upfield of their equatorial counterparts at 1.55 (H-3$_{eq}$/H-5$_{eq}$), 1.65 (H-4$_{eq}$) and 1.80 (H-2$_{eq}$/H-6$_{eq}$) ppm, and registered a more complex splitting pattern. Resonance for H-1 appeared at 3.30 - 3.42 ppm and overlapped with cysβ'. NMR assignments for the NAC and GSH moieties were consistent with those reported for other carbamoylated thiols (Han *et al.*, 1990; Mutlib *et al.*, 1990).

NMR spectroscopy was also instrumental in elucidating the regiochemistry of the 4- and (3-hydroxycyclohexyl)carbamoyl conjugates of GSH and NAC. 1,4-Cyclohexyl disubstitution of the (4-hydroxycyclohexyl)carbamoyl conjugates SFHG and NFHC gave rise to resonance at chemical shifts 1.20 - 1.45 ppm for unsubstituted axial protons, 1.87 ppm for unsubstituted equatorial protons and, 3.60 ppm for H-1 and H-4. (3-Hydroxycyclohexyl)carbamoyl conjugates registered a more complex resonance because of 1, 3-cyclohexyl disubstitution and the presence of a diastereomeric mixture. NMR assignments and C-H connectivity were elucidated with heteronuclear multiple quantum correlation (HMQC) spectroscopy (Figure 15). The appearance of cyclohexyl-derived $^{13}$C and
$^1$H resonance at more than one chemical shift was consistent with a diastereomeric mixture.

Resonance characteristic of the GSH moiety was similar to SFHG.

Figure 13. $^1$H-NMR spectrum of a carbamoylated NAC conjugate, typified in the case of $N$-acetyl-$S$-[(2-chloroethyl)carbamoyl]cysteine. Resonance signals at 3.17 and 3.45 ppm illustrate the resolution the cysteiny1 methylene protons.
Figure 14. COSY NMR spectrum of S-(cyclohexylcarbamoyl)glutathione (SCCG).

*Impurity. #Hydroxyl resonance of HOD.
Figure 15. $^{13}$C-$^1$H-HMQC NMR spectrum of $S$-[(3-hydroxycyclohexyl)carbamoyl]-glutathione (STHG). Correlations are designated $^{13}$C($^1$H).
3. Metabolism of nitrosoureas and nitrosourea-derived isocyanates

The metabolism of CCNU, BCNU and 2-chloroethyl isocyanate (CEIC) was investigated at the commencement of this project by direct, flow-injection APCI LC/MS. Metabolites were isolated from bile and urine by HPLC, and identified by comparison of their LC/MS and LC/MS/MS properties to synthetic reference compounds. As the capability for on-line through-column HPLC/electrospray MS became available, the scope of the research was expanded beyond qualitative identification of metabolite species to include quantitative determination of these compounds.

3.1. Metabolism of CCNU in rats and in humans

3.1.1. Identification of metabolites by APCI LC/MS and LC/MS/MS

Using the HPLC properties of SFCG, STCG and SCCG which eluted at tR 11.9, 18.8-19.7 and 26.5 min, respectively, the corresponding putative metabolites were purified from the bile of CCNU-dosed rats. Q1 scans for the putative metabolites SFHG and STHG depicted MH+ appearing at m/z 449, accompanied by what were considered to be the thermally-induced fragments at m/z 308 (GSH, RGS) and m/z 320 (Y2) (Figure 16) (see Table 3 for APCI-associated fragmentations). Comparison of the fragment ion spectrum of authentic SFHG and STHG to the CAD of the ion at m/z 449 from purified bile substantiated the identity of these compounds as the corresponding metabolites (Figure 17). In the Q1 scan of the putative metabolite SCCG, MH+ at m/z 433, was virtually sequestered in the spectral background, however, CAD of the pseudomolecular ion afforded a spectrum with fragments attributable to authentic SCCG (Figure 18).

APCI MS of the crude urine of CCNU-dosed rats shown in Figure 19 revealed the presence of prominent ions at m/z 289 and 305. Comparison of the CAD spectrum of an authentic standard of NCCC to the CAD spectrum of ion m/z 289 from urine supported the origin of the latter ion being due to the metabolite NCCC (Figure 20). Since m/z 305 was considered attributable to either NFHC, NTHC or both, HPLC purification was necessary to
distinguish these putative metabolites. HPLC purification of urine at the retention time of authentic standards allowed the putative metabolites NFHC (tR 8.2 min) and NTHC (tR 12.8 - 14.2 min) to be separated. Comparison of the MS/MS properties of synthetic NFHC and NTHC to the urine-derived ion at m/z 305, confirmed the identity of these compounds as metabolites of CCNU in the rat (Figure 21).

In an experiment of a design similar to that performed in the rat, a targeted search was performed for the metabolites NFHC, NTHC and NCCC in the urine of a patient on CCNU therapy. HPLC purification allowed the putative metabolites NFHC and NTHC to be discerned in their Q1 spectra with the appearance of MH⁺ at m/z 305 and (M+NH₄)⁺ at m/z 322. CAD of m/z 305, when compared to synthetic standards, confirmed the identity of NFHC and NTHC as urinary metabolites of CCNU in patient #91-20848.
Figure 16. APCI MS of the putative metabolite SFHG, with MH$^+$ at m/z 449, in the bile of CCNU-dosed rats at HPLC fraction t$_R$ 11.9 min. STHG was present in the HPLC fraction at t$_R$ 18.8 - 19.7 min, and appeared in a similar fashion with MH$^+$ at m/z 449.
Figure 17. APCI fragment ion spectra of (A) synthetic SFHG and (B) the putative metabolite SFHG in the bile of CCNU-dosed rats at HPLC fraction t_R 11.9 min., and present with MH^+ at m/z 449. STHG was present in the HPLC fraction at t_R 18.8 - 19.7 min, and displayed a similar APCI fragment ion spectrum.
Figure 18. APCI fragment ion spectra of (A) synthetic SCCG and (B) the putative metabolite SCCG in the bile of CCNU-dosed rats at HPLC fraction tR 26.5 min., and present with MH+ at m/z 433.
Figure 19. APCI MS of the putative metabolites NCCC and NFHC/NTHC, with MH$^+$ at m/z 289 and 305, respectively, in the urine of CCNU-dosed rats.
Figure 20. APCI fragment ion spectra of (A) synthetic NCCC and (B) the putative metabolite NCCC in the urine of CCNU-dosed rats with MH⁺ at m/z 289.
Figure 21. APCI fragment ion spectra of (A) synthetic NFHC and (B) the putative metabolite NFHC in the urine of CCNU-dosed rats at HPLC fraction $t_R$ 8.2 min., and present with $MH^+$ at m/z 305. NTHC was present in the HPLC fraction at $t_R$ 12.8 - 14.2 min, and displayed a similar APCI fragment ion spectrum.
3.1.2. Quantitation of CCNU metabolites by electrospray LC/MS/MS

The availability of on-line HPLC separation with electrospray MS detection afforded the opportunity to quantitate the urinary metabolites NFHC, NTHC and NCCC, and so assess the extent to which this pathway contributes to the metabolism of CCNU. Preliminary screening of urine using LC/MS Method 1 (with a relatively modest ramp in solvent B) allowed resolution of the coeluting cis- and trans-isomers of NFHC at t_R 17.2 - 17.8 min from the diastereomers of NTHC at ca t_R 18.4 - 19.2 min and NCCC at ca t_R 27 min (Figure 22). These compounds were detected by SRM for MH^+/R_GS at m/z 305/164 (NFHC and NTHC) and m/z 289/164 (NCCC). This analysis revealed a difference between rats and humans in the qualitative formation of the carbamoylated urinary metabolites of CCNU. Whereas NTHC was present as a diastereomeric mixture of at least three compounds in rats, this metabolite appeared as only one in human urine samples.

SRM analysis also revealed a mass spectral aberration at the m/z 305/164 transition at the retention time of NCCC. This signal was also apparent for synthetic NCCC when present as the pure compound, or as a mixture (Figure 23). No account could be made for this signal.

Metabolites were quantitated using LC/MS Method 4 (run time 44 min) which reduced the duration of LC/MS Method 1 (60 min) while still affording baseline separation of NFHC (t_R 13.7 min), NTHC diastereomers (t_R 14.4 - 14.6 min) and NCCC (t_R 18.3 min). The internal standard NCEC was monitored with the transition m/z 269/164 (MH^+/R_GS). NFHC (geometric isomers) and NTHC (diastereomers) were each quantitated collectively. Standard curves generated for NFHC (11.63 - 139.50 nmol/mL, r^2 = 0.997), NTHC (24.33 - 291.90 nmol/mL, r^2 = 0.994) and NCCC (13.10 - 157.20 nmol/mL, r^2 = 0.998) afforded very good correlation over the range of concentrations quantitated. Rats (n = 4) were dosed with CCNU (50 mg/kg), and urine collected for 24 h. Sample preparation prior to LC/MS analysis was minimal, requiring only to be spiked with internal standard and to be centrifuged to remove particulates. Urinary concentrations of NFHC, NTHC and NCCC and their recovery from the CCNU dose are detailed in Table 6. The rank order in the amount of each of the
carbamoylated NAC conjugates excreted in urine was NTHC > NCCC > NFHC, and together these metabolites accounted for 14.3 ± 2.9 % of the CCNU dose.

The urinary metabolite profile of carbamoylated NAC conjugates of two patients on CCNU therapy was also examined. NFHC, NTHC and NCCC, were identified in both patients but quantitation was performed in only the case where metabolite concentrations were within the limits of the already established standard curves. Concentrations for the metabolites were NFHC 117.5 nmol/mL, NTHC 124.1 nmol/mL, and NCCC 36.1 nmol/mL.

Table 6. Biotransformation of CCNU in rats and in humans to the urinary metabolites NFHC, NTHC and NCCC.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Conc. NFHC (nmol/mL)</th>
<th>Fraction of dose (%)</th>
<th>Conc. NTHC (nmol/mL)</th>
<th>Fraction of dose (%)</th>
<th>Conc. NCCC (nmol/mL)</th>
<th>Fraction of dose (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat 1</td>
<td>48.9</td>
<td>1.9 (5)</td>
<td>247.8</td>
<td>9.5 (2)</td>
<td>135.9</td>
<td>5.2 (12)</td>
</tr>
<tr>
<td>Rat 2</td>
<td>21.3</td>
<td>1.6 (6)</td>
<td>121.6</td>
<td>8.9 (7)</td>
<td>38.5</td>
<td>2.8 (4)</td>
</tr>
<tr>
<td>Rat 3</td>
<td>55.2</td>
<td>1.3 (8)</td>
<td>325.5</td>
<td>7.6 (26)</td>
<td>93.4</td>
<td>2.2 (5)</td>
</tr>
<tr>
<td>Rat 4</td>
<td>25.8</td>
<td>1.7 (12)</td>
<td>170.3</td>
<td>10.9 (3)</td>
<td>58.0</td>
<td>3.7 (3)</td>
</tr>
<tr>
<td>Mean</td>
<td>---</td>
<td>1.6 ± 0.3</td>
<td>---</td>
<td>9.2 ± 1.4</td>
<td>---</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>Patient 91-20848</td>
<td>117.5</td>
<td>---</td>
<td>124.1</td>
<td>---</td>
<td>36.1</td>
<td>---</td>
</tr>
<tr>
<td>Patient 90-24692</td>
<td>D, NQ</td>
<td>---</td>
<td>D, NQ</td>
<td>---</td>
<td>D, NQ</td>
<td>---</td>
</tr>
</tbody>
</table>

Rats were dosed with CCNU at 50 mg/kg and urine collected for 24 h post-dose. NAC conjugates were detected by SRM LC/MS/MS (see Figure 23). Values in parentheses are the coefficients of variation (%) associated with measurements performed in duplicate. The overall recovery of urinary NAC conjugates in rats is expressed as the mean (%) ± standard deviation (n = 4). Patients on CCNU therapy were administered the drug at 130 mg/m², and urine collected for 8 h post-dose. D, NQ = Detected but not quantitated.
Figure 22. LC/MS SRM for the transition MH+/RGS for the CCNU metabolites NFHC, NTHC and NCCC in the urine of (A) a dosed rat, and (B) a patient on chemotherapy. LC/MS Method 1 (see Experimental/Section 5.1.2.2). *Uncharacterized transition coincident with NCCC.
Figure 23. Typical LC/MS SRM for the transition $\text{MH}^+/R_GS$ for a standard mixture of NFHC, NTHC and NCCC. NCEC is the internal standard. LC/MS Method 4 (see Experimental/Section 5.1.2.2). *Uncharacterized transition coincident with NCCC.
3.2. Metabolism of BCNU and 2-chloroethyl isocyanate

3.2.1. Identification of metabolites by APCI LC/MS/MS

The metabolism of BCNU and CEIC to carbamoylated thiol conjugates was investigated with a targeted search for the potential metabolites SCEG and NCEC in the bile and urine, respectively, of dosed rats. Bile and urine were purified at the retention time of authentic standards of SCEG (tR 6.8 min) and NCEC (tR 9.4 min), respectively. The purified bile of rats revealed ion m/z 413 as the base peak in the Q1 scan (Figure 24). Considered attributable to MH⁺ of the biliary metabolite SCEG, this assignment was confirmed by comparison of its CAD spectrum to a synthetic standard of SCEG (Figure 25).

In like fashion, NCEC was identified as a urinary metabolite of both BCNU and CEIC. MH⁺ of the metabolite NCEC was present as ion m/z 269 in the Q1 scan of HPLC purified urine (Figure 26), and displayed a similar fragment ion spectrum to the synthetic reference compound (Figure 27).

3.2.2. Quantitation of NCEC in patients on BCNU therapy by electrospray LC/MS/MS

The carbamoylating activity of BCNU in humans was investigated by analyzing for NCEC as a urinary metabolite in a group of five patients on antineoplastic polytherapy (Table 7). Using the precursor ions at m/z 269 ([35Cl]MH⁺) and 271 ([37Cl]MH⁺), a targeted search was performed for NCEC by SRM of the transitions m/z 269/227, 269/164, 269/122 and 271/122 (Figure 28). These transitions appeared coincidentally at the retention time of an authentic standard of NCEC, 13.0 min (scan 297 - 298), and confirmed the existence of this compound as a metabolite of BCNU in humans.

Quantitation of NCEC in patient urine was performed by monitoring the MH⁺/RGSY₁ transitions m/z 269/122 and 289/122 for the metabolite and internal standard (NCCC), respectively (Figure 29), and the standard curve provided good correlation (r² = 0.988) in the concentration range 2.36 - 17.70 nmol/mL. Because the quantitation of NCEC was
performed solely with a view to establishing a range for concentration in patient urine, levels of the metabolite were not normalized. In the sampling of five patients studied urinary concentrations of NCEC varied from 5.0 to 13.6 nmol/mL (Table 7).

Table 7. Metabolism of BCNU to NCEC in patients on BCNU/cisplatin chemotherapy.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Conc. NCEC (nmol/mL)</th>
<th>Concomitant medication</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1: H.A.</td>
<td>5.0 (2)</td>
<td>dexamethasone, phenytoin, ondansetron</td>
</tr>
<tr>
<td>#2: M.H.</td>
<td>5.4 (4)</td>
<td>carbamazepine, diphenhydramine, ondansetron</td>
</tr>
<tr>
<td>#3: G.P.</td>
<td>8.4 (7)</td>
<td>dexamethasone, ondansetron, phenytoin</td>
</tr>
<tr>
<td>#4: M.N.</td>
<td>12.1 (8)</td>
<td>dexamethasone, phenytoin</td>
</tr>
<tr>
<td>#5: B.B.</td>
<td>13.6 (8)</td>
<td>dexamethasone, ondansetron, phenytoin</td>
</tr>
</tbody>
</table>

BCNU and cisplatin were administered by infusion over a three day period, and urine samples were taken at the mid-point of the dosage interval. NCEC was detected by SRM LC/MS/MS (see Figure 29). Values in parentheses are the coefficients of variation (%) associated with measurements performed in duplicate.
Figure 24. APCI MS of the putative metabolite SCEG, with MH$^+$ at m/z 413, in the bile of BCNU-dosed rats at HPLC fraction $t_R$ 6.8 min. SCEG was also present in CEIC dosed rats.
Figure 25. APCI fragment ion spectra of (A) synthetic SCEG and (B) the putative metabolite SCEG in the bile of BCNU and CEIC dosed rats at HPLC fraction $t_R$ 6.8 min, and present with MH$^+$ at m/z 413.
Figure 26. APCI MS of the putative metabolite NCEC, with MH⁺ at m/z 269, in the urine of BCNU-dosed rats at HPLC fraction tᵣ 9.4 min. NCEC was also present in CEIC dosed rats.
Figure 27. APCI fragment ion spectra of (A) synthetic NCEC and (B) the putative metabolite NCEC in the urine of BCNU- and CEIC-dosed rats at HPLC fraction tR 9.4 min, and present with MH⁺ at m/z 269.
Figure 28. LC/MS SRM screen for the urinary metabolite NCEC, $^{35}$ClMH$^+$ at m/z 269, from a patient on BCNU chemotherapy. LC/MS Method 2 (see Experimental/Section 5.1.2.2).
Figure 29. Typical LC/MS SRM quantitation of NCEC using the transition MH⁺/R₉SY₁.

NCCC is the internal standard. LC/MS Method 7 (see Experimental/Section 5.1.2.2).
4. Metabolism of $N$-formyl amphetamine to carbamoylated conjugates in rats

The metabolism of NFA was examined with a specific emphasis on characterizing the biliary and urinary metabolites which could arise as carbamoylation products of 1-methyl-2-phenylethyl isocyanate (MPIC). The procedure involved the combined use of LC/MS, LC/MS/MS and stable isotope methodology. NFA, prepared as a mixture of protio- and deuterio analogues ($^{2H}_0$NFA:$^{2H}_5$NFA, 50:50, w/w), was administered i.p. to rats and the bile and urine analyzed. A Q1 scan of bile was performed in the mass range m/z 250 to m/z 750 and the resulting TIC mass chromatogram (x-axis) and summed mass spectrum (y-axis) were constructed as a contour using SCIEX MacSpec3.2 software. The chromatographic run (acquisition duration 30 min) and mass range (500 a.m.u.) were divided into sectors as typified in Figure 30, in order to better identify isotopically related doublets as NFA derivatives. In the partial contour shown in Figure 30, four doublets at m/z 469, 474; m/z 283, 288; m/z 340, 345; and m/z 325, 330, each pair offset by 0.03 - 0.08 min, provided information on the presence of potential NFA-derived metabolites. In order to identify the structures of the compounds related to these ion doublets, CAD experiments were performed with each of the eight ions serving as the precursor species.

LC/MS/MS of the precursor ions MH$^+$ at m/z 469 and 474 (Figure 31) suggested the presence of $^{2H}_0$SMPG and $^{2H}_5$SMPG, respectively, consistent with the report of SMPG as a biliary metabolite of NFA (Mutlib et al., 1991). The ions m/z 179 and m/z 162 which were common to both fragment ion spectra were attributed to $R_G$SY$_2$ and $R_G$SZ$_2$, derived from the peptide backbone of GSH. Two fragments of the precursor ion m/z 469, namely ions m/z 237 and 340, carried information which was suggestive of their retention of the carbamoyl moiety, in that each of these was offset by 5 a.m.u. when derived from m/z 474. Accordingly, these isotopically-related fragments were consistent with $^{2H}_0$A$_2$Y$_2$, $^{2H}_5$A$_2$Y$_2$ (m/z 237, 242) and $^{2H}_0$Y$_2$, $^{2H}_5$Y$_2$ (m/z 340, 345).

CAD of the precursor ions MH$^+$ at m/z 283 and 288 revealed common fragments at m/z 76, 105 and 122 which were thought to account for $R_G$-CH$_2$, $R_G$S-OH and $R_G$S,
respectively, derived from the cysteiny1 backbone of the metabolite SMPC (Figure 32). Supporting evidence for the assignment of this structure to the metabolite was derived from the fragments which contained the aromatic nucleus. The phenyl ring-associated fragment ions m/z 91 and 119, arising from CAD of MH⁺ at m/z 283, paralleled the formation of ions m/z 96 and 124 proceeding from m/z 288.

The compounds in the contour with paired precursor ions MH⁺ at m/z 340, 345 and at m/z 325, 330 were not present at sufficient concentrations for a complete fragment ion spectrum to be obtained over the mass ranges selected viz. m/z 30 - 355 for CAD of m/z 340, 345, and m/z 30 - 340 for m/z 325, 330. Subdivision of these ranges to improve ion statistics allowed diagnostic fragments to be ascertained in a piecemeal fashion. Fragment ions m/z 179 and 162 were prominent for both precursor ions at m/z 340, 345 and were viewed as originating from the peptide backbone of a putative cysteiny1glycine conjugate of MPIC. SRM of the transitions m/z 340/179 and 340/162 coincident at 17.12 min, and m/z 345/179 and 345/162 coincident at 17.07 min substantiated the identities of [²H₀]- and [²H₅]SMPCG, respectively, in accordance with the observed precursor ion/fragment ion association, and the characteristic earlier retention time of the deuterio analogue before its protio partner (Figure 33). In an experiment of similar design, the putative metabolite NMPC was identified by monitoring of the transitions m/z 325/164 and 325/122, ascribed to MH⁺/R GS and MH⁺/R GS Y₁, respectively (Figure 34).

In order to validate the proposed structural assignments for each of the putative metabolites, authetic reference compounds were made available by synthesis. Comparison of the LC/MS properties of the biliary metabolites to the synthetic standards under SRM confirmed structural assignments (Figure 35). Screening of urine for the thiol conjugates by monitoring their corresponding transitions SMPG (m/z 469/179), SMPCG (m/z 340/179), SMPC (m/z 283/122) and NMPC (m/z 325/122) revealed the presence of only the NAC conjugate (Figure 36).
As a part of the screening process for carbamoylated metabolites an attempt was made to investigate the possible formation of \(N\)-[(1-methyl-2-phenylethyl)carbamoyl]glutathione (NMPG), the \(N\)-carbamoyl regioisomer of SMPG. LC/MS/MS analysis of synthetic SMPG and NMPG revealed that these compounds could be distinguished on the basis of MS properties (Figure 9) and chromatographic retention time (Figure 37). Under the HPLC conditions used, the \(S\)-carbamoyl- preceded the \(N\)-carbamoyl regioisomer by ca 0.20 min. Furthermore, SMPG fragment ions m/z 340 (Y\(_2\)) and 237 (A\(_2Y_2\)) were diagnostic for the \(S\)-conjugate, and so the transitions m/z 469/340 and 469/237 were monitored for this compound. On the other hand, compared to SMPG, NMPG favoured fragmentation to m/z 308 (R\(_G\)N), 291 (B\(_1\)) and 130 (R\(_G\)NB\(_1\)). Thus, the transitions m/z 469/308, 469/291 and 469/130 were monitored for NMPG. It should be noted, however, that these transitions are not entirely specific for NMPG and do appear, albeit at low intensity, for SMPG. Because of this, SRM LC/MS was useful in the analysis of SMPG and NMPG in a standard mixture (Figure 37). When this method was applied to bile, the possibility of NMPG being a metabolite could neither be supported nor ruled out with any degree of certainty because of the relatively high concentration of SMPG compared to the putative NMPG.
Figure 30. Partial LC/MS Q1 contour of the bile of rats dosed with $[^2\text{H}_0]\text{NFA}:[^2\text{H}_5]\text{NFA}$. This sector shows the appearance of the isotopically related doublets at $m/z$ 469, 474; $m/z$ 283, 288; $m/z$ 340, 345 and $m/z$ 325, 330.
Figure 31. LC/MS fragment ion spectrum of the biliary metabolites (A) [²H₀]SMPG, MH⁺ at m/z 469; and (B) [²H₅]SMPG, MH⁺ at m/z 474.
Figure 32. LC/MS fragment ion spectrum of the biliary metabolites (A) \([^{2}\text{H}_0]\)SMPC, MH\(^+\) at m/z 283; and (B) \([^{2}\text{H}_5]\)SMPC, MH\(^+\) at m/z 288.
Figure 33. LC/MS SRM for the biliary metabolites (A) $[^2H_0]SMPCG$, MH$^+$ at m/z 340; and (B) $[^2H_5]SMPCG$, MH$^+$ at m/z 345.
Figure 34. LC/MS SRM for the biliary metabolites (A) $[^2\text{H}_0]\text{NMPC}$, MH$^+$ at m/z 325; and (B) $[^2\text{H}_5]\text{NMPC}$, MH$^+$ at m/z 330.
Figure 35. (A) LC/MS SRM chromatograms of the putative biliary metabolites (a) SMPC, (b) NMPC, (c) SMPCG and (d) SMPG compared to (B) the corresponding synthetic reference compounds.
Figure 36. LC/MS SRM screening of urine for the metabolites (a) SMPC, MH⁺/RGS at m/z 283/122; (b) NMPC, MH⁺/RGSY₁ at m/z 325/122; (c) SMPCG, MH⁺/RGS at m/z 340/179; and (d) SMPG, MH⁺/RGSY₂ at m/z 469/179.
Figure 37. LC/MS SRM of a mixture of authentic standards of (A) SMPG and (B) NMPG.
5. *In vitro* metabolism of N-formylamphetamine by microsomes, intact mitochondria and mitoplasts

The metabolism of NFA to MPIC by rat hepatic fractions was investigated with an emphasis on characterizing the subcellular organelles responsible for the bioactivation of high molecular weight formamides. In view of the fact that microsomal P450 is a major participant in the biotransformation of NMF to MIC (Cross et al., 1990; Hyland et al., 1992), the metabolism of NFA by microsomes was to be used as a reference to which metabolism with intact mitochondria and mitoplasts could be compared.

Hepatic fractions were prepared from rats treated with phenobarbital (PB) or acetone, with untreated rats serving as control for both treatment groups. At the time of sacrifice, animals weighed 180 - 210 g. An administration protocol for acetone which involved a 5 % (v/v) solution in drinking water for 10 days (Song et al., 1989) was initiated. At the onset, animals weighed an average of 110 ± 9 g; however, it was evident by the third day of this regimen (average weight = 106 ± 8 g) that the animals were abstaining from both food and drink. The regimen was discontinued with the restoration of normal drinking water and rats rebounded to 133 ± 9 g on the fourth day. After the animals had acquired a weight of 182 ± 10 g, the protocol of oral gavage (Experimental/Section 5.2.1), a modification of the procedure used by Hyland et al. (1992), was implemented. Microsomes were prepared according to the procedure of Thomas et al. (1983) after isolation of the postnuclear fraction (Shayiq et al., 1991).

The total cytochrome P450 content of hepatic microsomes and mitoplasts is summarized in Table 8. Compared to microsomes, mitoplasts contained low levels of total P450 which was present in protein of a turbid nature. As a result, initial attempts to perform total P450 assays in mitoplasts, as previously described for microsomes (Panesar, 1993), with the Hewlett-Packard Diode Array 8452A Spectrophotometer, were unsuccessful. The use of second derivative spectrophotometry (Ghersi-Egea et al., 1987), a strategy used to deal with total P450 assays in turbid systems, was also unsatisfactory. The SLM AMINCO DW-2®
double-beam spectrophotometer proved to be sufficiently sensitive for the low P450 levels in mitoplasts, and robust in correcting for the particulate nature of this subcellular fraction. Consequently, this instrument was used for all total P450 assays.

Table 8. Total P450 content of rat hepatic microsomes and mitoplasts, spectrally quantitated at the absorbance of the CO difference spectrum of the dithionite-reduced enzyme.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Cytochrome P450 content (nmol-P450/mg-protein)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microsomes</td>
<td>Mitoplasts</td>
</tr>
<tr>
<td>Untreated</td>
<td>1.05 (2)</td>
<td>0.13 (23)</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.22 (6)</td>
<td>0.06 (17)</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>2.22 (6)</td>
<td>0.10 (10)</td>
</tr>
</tbody>
</table>

Microsomes and mitoplasts were pooled from twelve rats per treatment group. Values are expressed as the mean of determinations performed in triplicate and their associated coefficients of variation in parentheses.

5.1. Microsomal desaturation of N-formyl amphetamine to 1-methyl-2-phenylethyl isocyanate

The microsomal conversion of NFA to MPIC was monitored in the presence of GSH to trap the isocyanate product in the form of its thiocarbamate conjugate SMPG (Scheme 17). The reaction was terminated by the addition of TFA, and under the ensuing acidic conditions, SMPG was a stable chemical species readily amenable to quantitation by LC/MS/MS. SRM pairing of the MH⁺/Y₂ transitions at m/z 469/340 and 474/345 for SMPG and the internal standard [²H₅]SMPG, respectively (Figure 38) provided a sensitive and specific assay with good correlation (r² = 0.992) for the GSH conjugates over the concentration range 1.84 - 36.8 nmol/mL.
Scheme 17. Microsomal metabolism of NFA to MPIC which is trapped by GSH in the form of SMPG for analysis by LC/MS/MS.

Incubation conditions for microsomes with NFA were in accordance with previously reported methods for the metabolism of NMF (Cross et al., 1990; Hyland et al., 1992). Using 1.0 nmol of spectrally determined P450 from microsomes and 5 mM NFA, the formation of SMPG was determined at designated time points to characterize the duration in which the rate of formation of product was linear (Figure 39). The rate of formation of SMPG over 10 min was linear for all treatment groups, and so, this 10 min duration was used for all subsequent microsomal incubations with NFA.

The formation of SMPG from NFA was dependent on viable microsomes and NADPH, since no reaction occurred in the presence of heat-inactivated microsomes and in the absence of the cofactor (Table 9). Probing of the biotransformation with the broad spectrum P450 inhibitor metyrapone revealed a 63 % reduction in SMPG formation. The possible involvement of Fenton-type P450 catalysis, investigated by the addition of catalase to neutralize H$_2$O$_2$, revealed a marginal 15 % inhibition in metabolite formation.
Table 9. Metabolism of NFA to SMPG by microsomes prepared from untreated rats.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>SMPG (nmol/min/nmol-P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete system</td>
<td>1.22 ± 0.16 (6)</td>
</tr>
<tr>
<td>boiled microsomes</td>
<td>ND (2)</td>
</tr>
<tr>
<td>NADPH absent</td>
<td>ND (2)</td>
</tr>
<tr>
<td>complete system + metyrapone (1.0 mM)</td>
<td>0.45 ± 0.01 (3)</td>
</tr>
<tr>
<td>complete system + catalase (1000 units)</td>
<td>1.04 ± 0.02 (3)</td>
</tr>
</tbody>
</table>

Values are expressed as the mean ± standard deviation derived from multiple experiments using microsomes pooled from twelve untreated rats. Numbers in parentheses indicate the number of separate experiments from which the mean value was derived. ND = not detected (< 0.04 nmol/min/nmol-P450).

5.1.1. Investigation into the effect of inducers and mechanism-based inhibitors on microsomal NFA desaturation

At the onset of our investigation into the microsomal metabolism of NFA, we considered P450 2E1 as potential catalyst, consistent with the involvement of this isoform in bioactivation of NMF to MIC (Hyland et al., 1992). P450 2B1/2B2, an isoform which constitutes part of our research interests in valproic acid bioactivation (Panesar, 1993), was included in this study for comparative assessment alongside P450 2E1 for our probe into the P450s that may catalyze NFA metabolism.

Microsomes were prepared from rats treated with acetone and PB in accordance with their induction of P450 2E1 (Johansson et al., 1988) and P450 2B1/2B2 (Waxman and Azaroff, 1992), respectively. Microsomal conversion of NFA to SMPG was elevated (by 25 %) only in the case of PB treatment (Figure 40 A).

Orphenadrine (25 µM) and DEDTC (500 µM) were employed as mechanism-based inhibitors to probe the involvement of P450 2B1/2B2 (Murray and Reidy, 1990) and P450 2E1 (Guengerich et al., 1991), respectively. The inhibition of SMPG formation by orphenadrine in microsomes from untreated and PB treated rats was marginal (Figure 40 A).
Modest levels of inhibition of NFA metabolism were also exhibited by DEDTC in microsomes from untreated (40 %) and acetone-treated (22 %) rats (Figure 40 A).

5.1.2. Metabolism of the model P450 substrates p-nitrophenol and pentoxyresorufin compared to NFA in microsomes from untreated rats, and acetone and PB treated rats

The conversion of NFA to SMPG in rat liver microsomes was compared to model isozyme-selective biotransformations, namely p-nitrophenyl hydroxylase (PNH) for P450 2E1 (Reinke and Moyer, 1985; Koop, 1986) and pentoxyresorufin O-dealkylase (PROD) for P450 2B1/2B2 (Burke et al., 1985). Microsomal PNH activity in untreated rats (0.47 nmol/min/nmol-P450) was elevated 2-fold in acetone treated rats, and appeared to be reduced in rats treated with PB (Figure 40 B). DEDTC inhibited PNH activity for all microsomal preparations with the effect being most pronounced (96 %) in microsomes from rats treated with acetone. Orphenadrine, unlike DEDTC, exerted no inhibition of PNH activity.

PROD activity (0.19 nmol/min/mg-protein) in microsomes prepared from untreated rats was dramatically elevated with PB (19-fold) and acetone treatment (8-fold) (Figure 40 C). Orphenadrine was inhibitory toward PROD activity to the extent of 98 % and 88 % in microsomes prepared from PB and acetone-treated rats, respectively. Although not exerting as strong an influence as orphenadrine, DEDTC also inhibited PROD activity in the microsomes of rats prepared from PB (75 %) and acetone (78 %) treated rats. In light of the observed inhibition of P450 2B1/2B2-selective PROD activity by DEDTC, a reported mechanism-based inhibitor of P450 2E1, we were prompted to more closely evaluate the influence of DEDTC concentration on PROD catalysis in microsomes prepared from PB and acetone-treated rats (Figure 41). PROD activity decreased with DEDTC concentration. Even the lowest concentration examined (100 µM) exerted ca 32 % inhibition of the original activity in microsomes from both PB and acetone treated rats.
5.2. An investigation into amphetamine as a possible product of NFA formyl metabolism

During P450-catalyzed biotransformation of NFA, it is possible that the radical intermediate which gives rise to MPIC could also afford amphetamine as an ancillary product from a divergent catalytic pathway (see Discussion/Section 4.2). Described here is a preliminary account of an approach to the assay for amphetamine. Amphetamine standards and microsomal aliquots were spiked with two internal standards, namely [²H₅]SMPG and phentermine, and extracted under basic conditions (Experimental/Section 5.2.3.1). GC/MS analysis involved selected monitoring of the ions m/z 118, 123 and 132 for amphetamine, [²H₅]amphetamine and phentermine, respectively (Figure 42). A standard curve generated for amphetamine concentrations over the range 0.46 - 36.80 nmol/mL demonstrated good correlation ($r^2 = 0.989$). As illustrated with the recovery of [²H₅]amphetamine from a standard mixture, it was apparent that [²H₅]SMPG undergoes decomposition during the course of sample preparation for GC/MS. Inferentially, any SMPG formed as a microsomal product was also likely to contribute to amphetamine present in incubation mixtures. Furthermore, analysis of incubations with denatured microsomes in which no detectable SMPG was present (vide supra) revealed the presence of amphetamine as a minor contaminant of NFA. Consequently, the present procedure did not permit an unequivocal demonstration of the formation of amphetamine as a metabolite of NFA.
Figure 38. Typical LC/MS SRM for the transitions MH⁺/Y₂ for a standard solution of (A) SMPG spiked with (B) the internal standard [²H₅]SMPG. LC/MS Method 5 (see Experimental/Section 5.2.3.1) was applied to the quantitation of SMPG in microsomal incubations.
Figure 39. Time course for the formation of SMPG as a product of NFA by hepatic microsomes prepared from untreated, phenobarbital and acetone-treated rats. Values are expressed as the mean of two determinations using microsomes pooled from twelve rats per treatment group.
Figure 40. Effects of phenobarbital (PB) and acetone treatment on rat hepatic microsomal activities: (A) SMPG formation (NFA formyl desaturation) compared to (B) p-nitrophenyl hydroxylase and (C) pentoxyresorufin O-dealkylase (Experimental/Section 5.2.3 - 5.2.4). The inhibition of microsomal catalysis of these reactions by orphenadrine (25 μM) and DEDTC (500 μM) was compared. Values are expressed as the mean ± standard deviation of two (B, C) to three (A) separate experiments using microsomes pooled from twelve rats per treatment group. * These experiments were not performed.
Figure 41. Effect of DEDTC on the PROD activity of microsomes prepared from acetone and PB treated rats. Values are expressed as the mean ± standard deviation of two separate experiments using the microsomes pooled from twelve rats per treatment group.
Figure 42. GC/MS analysis of a standard mixture of amphetamine, $^2$H$_5$SMPG and phentermine, extracted under alkaline conditions and derivatized as described in Experimental/Section 5.2.3.1). HFBA derivatives of (A) amphetamine, (B) $^2$H$_5$amphetamine and (C) phentermine were detected by SIM of m/z 118, 123 and 132, respectively. The fragmentation shown is in accordance with that proposed by Gjerde et al. (1993).
5.3. Mitochondrial bioactivation of N-formylamphetamine

The hypothesis that NFA bioactivation to MPIC could be a mitochondrial phenomenon was investigated in intact mitochondria and in sonicated mitoplasts. Metabolism in mitochondria was supported by glutamate/malate and in mitoplasts by NADPH.

5.3.1. NFA metabolism in intact mitochondria

Mitochondria are intrinsically fragile organelles and so their integrity was routinely assessed after preparation. Mitochondrial integrity was evaluated either by the performance of oxidative phosphorylation (Estabrook, 1967) or by the release of the intramitochondrial marker citrate synthase upon sonication (Shepherd and Garland, 1969). Mitochondria which were judged to be intact exhibited (1) respiratory control ratios of 5.1 to 12.0 and ADP/O ratios of 2.3 to 3.1 with glutamate/malate as substrate (Figure 43) or (2) ≥96 % release of citrate synthase activity.

The bioactivation of NFA to MPIC in mitochondrial incubations was accomplished by trapping the isocyanate as SMPG. Screening for SMPG in mitochondrial incubations was approached by SRM of the transitions m/z 469/340 (MH+/Y2) and m/z 469/237 (MH+/A2Y2). LC/MS data pointed to the formation of SMPG as a metabolite with the coincident response of these transitions, and although some interference was apparent at the transition m/z 469/340, a much improved response in ion current and signal/noise ratio was achieved with the m/z 469/237 transition (Figure 44A). Incubations performed in the absence of mitochondria (Figure 44B) and in the absence of substrate (Figure 44D) failed to yield this compound. Spiking of the productive mitochondrial incubation with [2H5]SMPG (tR 5.85 min) corroborated the identity of the metabolite (tR 5.91 min) by having a slightly shorter retention time.
Figure 43. Typical oxygraph trace showing the performance of oxidative phosphorylation by intact mitochondria. Arrows indicate the addition of ADP. Mitochondria exhibited respiratory control ratios of 5.1 to 12.0 and ADP/O ratios of 2.3 to 3.1 with glutamate/malate as substrate. Consequently, these organelles were considered to be well-coupled (Estabrook, 1967) (see Experimental/Section 5.2.4.4).
Figure 44. LC/MS SRM screening for SMPG as a mitochondrial metabolite of NFA. (A) Complete system; (B) incubation without mitochondria; (C) complete system spiked with $[^2H_5]$SMPG; (D) incubation without NFA.
5.3.2. NFA metabolism in sonicated mitoplasts

In order to investigate the metabolism of NFA by mitochondrial P450 when supported by NADPH, mitochondria were treated with digitonin to remove the outer mitochondrial membrane and contaminating microsomal adherence (Shayiq *et al.*, 1991). Because of the absolute dependence of mitochondrial P450 on the matrix soluble ferredoxin/ferredoxin reductase electron transport system, it was necessary to evaluate mitoplast intactness to ensure that these enzymes were retained during the course of preparation. Membrane integrity (≥96 %) was assessed by the release of citrate synthase (Experimental/Section 5.2.4.1). Mitoplasts, although intact, tended to be inferiorly coupled (compared to mitochondria) and so the performance of oxidative phosphorylation was not an appropriate test for mitoplast intactness.

An evaluation of microsomal contamination in mitoplasts, summarized in Table 11, was derived from the activity of KCN–insensitive microsomal NADPH-cytochrome c reductase and immunochemically detected P450 2B and 2C isoforms (Figure 45). Shayiq and Avadhani (1990) reported the occurrence of a PB inducible mitochondrial P450, termed P450mt4, which was closely related to P450 2B1 with respect to both electrophoretic mobility (when subjected to SDS PAGE with 12 % acrylamide), and immunological response (when probed on a Western blot with anti-P450 2B1 IgG).

An attempt was made to electrophoretically distinguish between the putative P450mt4 and possible contaminating P450 2B1 in mitoplasts prepared from PB-treated rats by using SDS PAGE with 7.5 % acrylamide instead of 12 % used by Shayiq and Avadhani (1990). Probing of the resulting Western blot with anti-P450 2B1 IgG did not unmask the putative P450mt4, but instead revealed the presence of two bands for mitoplasts in lanes 7 and 8 coincident with the isoforms P450 2B1 and 2B2 present in microsomes in lanes 9 - 11 (Figure 45 A). In light of this observation, we elected to exploit the immunological detection of what we considered to be contaminating P450 2B2 in mitoplasts, because no reports in the literature document the existence of mitochondrial P450s with similar electrophoretic
migration and immunochemical properties to P450 2B2. Values of Integrated Intensity/mg-protein for the detected bands were used to obtain a relative evaluation of microsomal contamination in mitoplasts from rats treated with PB. Comparison of the Integrated Intensity of the P450 2B2 band allowed the contamination of microsomes in mitoplasts to be calculated (Table 10).

In the microsomes of untreated rats, P450 2B1 was undetectable, and present at low levels were P450 2B2 and a noninducible P450 2B isoform with a longer electrophoretic migration, considered to be P450 2B3. P450 2B1, 2B2 and 2B3 were also detected in microsomes from acetone treated rat, but only at the 0.4 μg-protein loading. Thus, immunologically probing with anti-P450 2B1 IgG was unsuitable for evaluating the microsomal contamination of mitoplasts from untreated and acetone-treated rats. In order to address this issue, we resorted to the use of polyspecific anti-P450 2C11 IgG which was directed toward P450 2C isoforms constitutively expressed in the male rat. This antibody was known to cross-react with P450 2C6, 2C7 and 2C13, and was considered an ideal probe for the screening of microsomal contamination in the mitoplasts from all treatment groups in a single run. Five immunodetectable bands, most readily distinguished at the 0.4 μg loading of microsomes obtained from acetone-treated rats (lane 16), were termed I, II, III, IV and V (Figure 45 B). The coincident migration of a purified standard of P450 2C11 with band III allowed only this immunodetected protein to be assigned. Qualitative evaluation of the blot allowed some modulation of P450 isozymes to be discerned for microsomal proteins. For example, comparison of lanes 5 & 10, 5 & 15, and 6 & 16 revealed that the level of proteins in bands I and II were elevated by PB and acetone treatment. The relative amount of immunodetectable P450 2C11 in the mitoplasts and microsomes of each treatment group was used to calculate the microsomal contamination in mitoplasts (Table 10).

Sonicated mitoplasts derived from untreated, PB-treated and acetone-treated rats catalyzed the NADPH-dependent metabolism of NFA to SMPG. SMPG was quantitated as described for microsomal incubations and results are summarized in Table 11.
Figure 45. Western blot analysis of mitoplast and microsomal fractions isolated from the livers of untreated, PB treated and acetone treated rats. Proteins separated by SDS PAGE were electrophoretically transferred on to nitrocellulose and sequentially probed with (A) anti-P450 2B1 IgG (2.0 μg/mL) or (B) anti-P450 2C11 IgG (40 μg/mL) and a secondary horseradish peroxidase conjugated antibody. Immunologically recognized proteins were detected by ECL™ methodology. Lane assignments, indicating the total amount of P450 or protein loaded per well, are as follows: (A) 1: P450 2B1 standard, 0.025 pmol; 2: untreated mitoplasts, 2 μg; 3: untreated mitoplasts, 4 μg; 4: untreated microsomes, 0.04 μg; 5: untreated microsomes, 0.1 μg; 6: untreated microsomes, 0.4 μg; 7: PB mitoplasts, 2 μg; 8: PB mitoplasts, 4 μg; 9: PB microsomes, 0.04 μg; 10: PB microsomes, 0.1 μg; 11: PB microsomes, 0.4 μg; 12: acetone mitoplasts, 2 μg; 13: acetone mitoplasts, 4 μg; 14: acetone microsomes, 0.04 μg; 15: acetone microsomes, 0.1 μg; 16: acetone microsomes, 0.4 μg; 17: P450 2B1 standard, 0.025 pmol-P450. Assignments for (B) are similar to (A) except for lanes 1 and 17: P450 2C11 standard, 0.063 pmol.
Table 10. Evaluation of microsomal contamination in rat hepatic mitoplasts based on the marker enzyme KCN-insensitive NADPH-cytochrome c reductase and immunodetectable P450 2B2 and 2C11.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>NADPH-cytochrome c reductase</th>
<th>Immunodetectable microsomal P450 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity (%)</td>
<td>Specific activity (%)</td>
</tr>
<tr>
<td>untreated</td>
<td>0.15</td>
<td>5.6</td>
</tr>
<tr>
<td>acetone</td>
<td>0.10</td>
<td>4.6</td>
</tr>
<tr>
<td>phenobarbital</td>
<td>0.21</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Contamination based on total activity was calculated using the total activity of the marker in both mitoplast and liver homogenate fractions. Contamination based on specific activity was calculated using the specific activity of the marker in the mitoplast and microsomal fractions (see Experimental/Section 5.2.4.3). Contamination based on immunodetection was calculated using the specific Integrated Intensities of mitoplast and microsomal protein detected on Western blots (see Experimental/Section 5.2.5). ND = not detected.

Table 11. SMPG formation by mitoplasts prepared from untreated, PB-treated and acetone-treated rats.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SMPG (mmol/μmol-P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>untreated</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>phenobarbital</td>
<td>0.57 ± 0.06</td>
</tr>
<tr>
<td>acetone</td>
<td>0.93 ± 0.12</td>
</tr>
</tbody>
</table>

Incubations were conducted for 90 min with sonicated mitoplasts in the presence of NADPH as cofactor (see Experimental/Section 5.2.3.3). Mitoplasts were pooled from twelve rats per treatment group. Values are expressed as the mean ± standard deviation of determinations performed in duplicate.

6. Evaluation of the reactivity and toxicity of thiocarbamate conjugates

Under physiological conditions thiocarbamates decompose to release the isocyanate and the free thiol (Baillie and Slatter, 1991). The following experiments were designed to investigate the equilibrium associated with thiocarbamate formation, and the toxic effects associated with this process in selected enzyme activities of isolated mitochondria.
6.1. Chemical decomposition and reactivity of GSH and NAC conjugates of 2-chloroethyl isocyanate

The decomposition of SCEG was examined in phosphate buffer (pH 7.4) at 37 °C. HPLC analysis of aliquots withdrawn at designated time points over an interval of 6.4 h allowed the concentration time course of SCEG to be followed. A plot of the first order decomposition curve log [SCEG] vs time gave a slope of -0.0010, and allowed the half-life of SCEG to be calculated at 301 min (5.0 h) (Figure 46).

A direct consequence of thiocarbamate decomposition is that the released isocyanate is capable of reacting with other nucleophiles present. We sought to investigate this event by observing SCEG (1 mM) decomposition in the presence of NAC (10 mM). Under these conditions, SCEG decomposed with a half-life of 44 min, accompanied by the formation of NCEC (Figure 47A). In an experiment of similar design NCEC (1 mM) decomposed in the presence of GSH with a half-life of 3.7 h with the concomitant formation of SCEG (Figure 47B).

The reactivity of thiocarbamates with other nucleophiles in vitro directed our focus toward the examination of this phenomenon in vivo. NCEC was administered to rats and, based on the conjecture that this compound would react with endogenous GSH to afford SCEG, a targeted search was made for the GSH conjugate as a biliary metabolite. SRM using LC/MS Method 4 (Experimental/Section 5.1.2.2) was performed for transitions proceeding from MH+ at m/z 413. Precursor ion/fragment ion pairs m/z 413/284(Y2), 413/181(A2Y2) and 413/179(RGSY2) revealed the presence of SCEG (tR 12.33 min), detected along with the dose of NCEC (tR 13.89 min) with the transitions m/z 269/164(RG S) and 269/122(RG SY1) (Figure 48). Comparison of the latter chromatogram to that of a mixture of authentic NCEC and SCEG supported the assertion that these compounds were present in the bile of NCEC-dosed rats.
Figure 46. First order decomposition curve of SCEG in phosphate buffer (pH 7.4) at 37 °C (see Experimental/Section 5.2.6.1).
Figure 47. (A) Decomposition of SCEG (1 mM) in the presence of phosphate buffered NAC (10 mM, pH 7.4) at 37 °C with the concomitant formation of NCEC. (B) Decomposition of NCEC (1 mM) in the presence of phosphate buffered GSH (10 mM, pH 7.4) at 37 °C with the concomitant formation of SCEG (see Experimental/Section 5.2.6.1).
Figure 48. LC/MS SRM chromatograms of (A) the putative biliary components (a) SCEG and (b) NCEC, compared to (B) authentic standards of these compounds.
6.2. Inhibitory action of SCEG and SMG toward mitochondrial enzyme activities

The intrinsic reactivity of thiocarbamate conjugates prompted an investigation of the toxic effect of these compounds on enzymes. Because of the key role played by mitochondria in cellular function, it seemed appropriate to use activities of these organelles as a model (Experimental/Sections 5.2.6.2 - 3).

In the presence of 1 mM SCEG, mitochondrial GR activity declined rapidly to ca 10 % of control levels in 7 min, and remained depressed for up to 6.25 h. At 6.25 h mitochondria were evaluated for their release of citrate synthase, and found to be 96 % intact (Figure 49). Citrate synthase was itself inhibited by SCEG by only 10 % of control activity and was therefore considered a suitable marker for this experiment.

Mitochondrial respiration was evaluated in the performance of oxidative phosphorylation. After a 90 min incubation period, state III respiration was apparent in control mitochondria as an ADP-induced oxidative burst with ADP/O ratios of 2.4 to 3.2, and respiratory control ratios of 3.0 to 7.2; however, this process was blocked in mitochondria exposed to SMG (3.1 mM) (Figure 50).

Thus, it is apparent from these findings that GR and oxidative phosphorylation are two mitochondrial activities which could be targeted by thiocarbamates that arise as metabolites of nitrosoureas and formamides.
Figure 49. Time course for the inhibition of mitochondrial glutathione reductase by SCEG. Mitochondria were pooled from four rats and GR activity determined for test and control experiments as described in Experimental/Section 5.2.6.2). Values are recorded as the mean % change ± standard error of separate test experiments (n = 3) compared to control experiments (n = 2).
Figure 50. Oxygraph trace showing the effect of SMG (3.1 mM) on mitochondrial respiration using glutamate/malate as substrate. Mitochondria were incubated in buffered system (pH 7.6) containing sucrose, mannitol, HEPES, EDTA for 90 min. Arrows indicate the addition of ADP. Trace A shows a control incubation. ADP/O ratios were 2.4 - 3.2 and respiratory control ratios 3.0 - 7.2. Trace B shows inhibition of state III oxidation by SMG (see Experimental/Section 5.2.6.3).
IV. DISCUSSION

1. Analysis of GSH and NAC conjugates

GSH and NAC metabolites, by virtue of their being conjugated to transient, reactive species, provide valuable insight into the chemical nature of these intermediates (Ketterer and Mulder, 1990). However, the polar, thermolabile and often chemically unstable nature of these thiol conjugates restricts their amenability to few analytical techniques. LC/MS and NMR spectroscopy are two such techniques, and these analytical methods were utilized here for probing the structural identity of several thiol conjugates. Whereas NMR spectroscopy was used solely for the characterization of synthetic compounds, LC/MS was the mainstay in the identification and quantitation of compounds of both synthetic and biological origin.

In these studies, LC/MS was conducted using either thermospray or API methods of ionization. Sufficient use was made of thermospray LC/MS to allow some of the features of this technique to be documented. The ionization process was found to be sensitive to subtle nuances in operational parameters, including interface temperatures, and mobile phase flow rate and composition, a feature which has been noted by others (Covey et al., 1986; Straub, 1988). Ionization allowed some conservation of the pseudomolecular ion to be accomplished in the case of GSH (Figure 11); however, considerable fragmentation of the NAC conjugate NFHC occurred (Figure 12). These results are in line with the fact that the ionization of thiol conjugates under thermospray is considerably compound-dependent (Parker et al., 1988), and although GSH and NAC conjugates were not compared here, the latter (Conchillo et al., 1988) tend to fragment less than their GSH counterparts (Beattie and Blake, 1989; Rashed et al., 1989; Dulik et al., 1990). Rather than a mass spectrometric phenomenon, these fragmentations are considered attributable to thermal effects (Parker et al., 1988). Successful strategies in promoting the MH+ ion have employed esterification (Conchillo et al., 1988; Baillie et al., 1989) and post-column addition of organic modifier (Bean et al., 1990).
Compared to thermospray, API LC/MS displayed superior versatility in the analysis of a range of structurally different thiol conjugates under standard ionization conditions. Apparent under APCI conditions, however, was the propensity to thermal fragmentation which is intrinsic to this technique (Avery et al., 1992; Kasuya et al., 1992). This was more apparent in the GSH conjugates than in the NAC conjugates. GSH conjugates underwent loss of the glutamyl moiety to afford the Y2 ion and loss of the substrate entity to afford RGS (GSH, m/z 308) (Table 3). Fragmentation to GSH promoted the cascade MH+ → RGS (m/z 308) → RGS-H2O (m/z 290) → RGS-H2O-H2S (m/z 256) which appears to proceed with the preliminary loss of H2O as evidenced by the absence of the fragment RGS-H2S (m/z 274). Interestingly, the GSH-related ions m/z 256 and 290 appeared unique to APCI thermally-induced fragmentation and were not observed under CAD conditions.

Strong conservation of the pseudomolecular ion under API allowed MS/MS techniques to be utilized to advantage in these studies for the identification and quantitation of thiol conjugates. CAD of the pseudomolecular ion of the GSH conjugates studied here proceeded with a high abundance of fragments derived from the GSH moiety namely, RGSY2 (cysteinylglycine, m/z 179) and RGSZ2 (m/z 162) (Table 4). However, these fragments are not characteristic for all carbamoylated GSH conjugates, being observed in some conjugates (Han et al., 1990), but not in others (Jin et al., 1994). Fragments with the carbamoyl linkage, Y2 and A2Y2, carried a considerable portion of the ion current, which has been noted by others as a feature among GSH conjugates (Haroldsen et al., 1988; Jin et al., 1994). Particularly noteworthy in the fragment Y2 is the characteristic loss of 129 a.m.u. This was highlighted in a recent review (Baillie and Davis, 1993) as one of three screening strategies for GSH conjugates and the technique has been exploited by several researchers (Ballard et al., 1990; Davis et al., 1993; Kassahun et al., 1993). Another screening technique involves precursor scanning of m/z 308 (GSH, RGS). Under the CAD conditions used here (collision energy 31 eV), RGS fragmentation carries minimal ion current; however, this fragmentation pathway is considerably promoted at higher collision cell voltages (Murphy et al., 1992).
CAD of NAC conjugates afforded R_GS (m/z 164) and R_GSY_1 (m/z 122) as major fragments (Table 5), the former of which is demonstrably class characteristic (Deierding et al., 1989; Jones et al., 1993). As a matter of fact, the precursor ion scanning of m/z 164 has been proposed as a screening method for NAC conjugates and has been utilized for this purpose (Davis et al., 1993). The substrate entity of the NAC conjugates examined here was modestly predisposed to carriage of the ion current. Although this fragmentation pathway does not appear to contribute significantly under positive ionization conditions, the situation could be reversed under negative ionization conditions (Jones et al., 1993).

2. Metabolism of nitrosoureas

The metabolic fate of the nitrosoureas CCNU and BCNU in rats and in humans was investigated with a view to characterizing the nature of the carbamoylating species released upon in vivo transformation of these compounds. Because nitrosourea-derived isocyanates are unstable, we sought to gain information on the carbamoylation event through the characterization of GSH and NAC conjugates excreted in bile and urine, respectively.

2.1. Metabolism of CCNU in rats and in humans

In the initial phase of this project, mass spectrometry of thiol conjugates was conducted through a contract laboratory and performed on a SCIEX API III instrument set primarily for APCI LC/MS. In order to circumvent the high cost of time required to develop methods for through-column HPLC-MS/MS at this location, we opted to purify putative metabolites from bile and urine beforehand by HPLC and analyze these fractions by direct flow-injection LC/MS/MS (Figures 16 - 21). The purpose of prior HPLC purification was twofold. Firstly, metabolites were, to a considerable extent, freed of the interfering biological matrix and secondly, positional isomers, as in the case of SFHG/STHG and NFHC/NTHC, could be distinguished. The development of HPLC conditions for the purification of putative metabolites was facilitated by the availability of standard compounds prepared by synthesis.
Our identification of carbamoylated thiol conjugates as products of CCNU in rats and in man using APCI LC/MS/MS which has since been published (Borel and Abbott, 1993) was a novel finding in that it represented for the first time structurally characterized metabolic species which provide evidence for the in vivo carbamoylating activity of nitrosoureas. In rats, 4-hydroxycyclohexyl, 3-hydroxycyclohexyl and cyclohexyl isocyanate were trapped and identified in the form of their GSH conjugates in bile and as NAC conjugates in urine. In the case of the patient, the NAC conjugates of 4-hydroxycyclohexyl and 3-hydroxycyclohexyl isocyanate were identified as urinary metabolites.

With the availability of through-column HPLC electrospray MS/MS in the latter phase of this project, we were able to expand the scope of our studies to involve more sensitive SRM LC/MS/MS analyses, and do so quantitatively (Figures 22 - 23, Table 6). Studies on CCNU metabolism which focussed on the urinary carbamoylated NAC conjugates confirmed the formation of NFHC, NTHC and NCCC as metabolites of CCNU in rats. HPLC conditions using a modest gradient increase in organic modifier (LC/MS Method 1) resulted in coelution of NFHC geometric isomers and separation of NTHC diastereomers (Figure 22). Because a synthetic standard was unavailable for the potential metabolite N-acetyl-S-[(2-hydroxycyclohexyl)carbamoyl]cysteine, this conjugate was targeted by searching for a response in the transition m/z 305/164 other than for those associated with NFHC and NTHC. No such signal was detected in the urine of rats which suggests two possibilities. Either, the sought after NAC conjugate was below the limit of detection under the present method, consistent with the fact that 2-hydroxycyclohexyl isocyanate derivatives were absent in the urine of CCNU-dosed rats (Kohlhepp et al., 1981) (vide infra), and/or this metabolite coeluted with NFHC or NTHC.

What did become apparent during the course of SRM analyses was a response at the retention time of NCCC (MH+ at m/z 289) which carried an ion transition m/z 305/164. Injection of pure synthetic standards for the NAC conjugates revealed that this signal was surprisingly originating from NCCC. Furthermore, this transition remained associated with
NCCC under different HPLC conditions (LC/MS Methods 1 and 4), carried a similar chromatographic profile, and was present in a mixture of standards (Figure 23) and in urine samples from both rats and humans (Figure 22). The delayed retention time of this chromatographic peak, compared to NFHC and NTHC, argues against this response being ascribed to a hydroxylated species. Because this peak appears to be attributable to NCCC, its origin is likely to be mass spectrometric, and an adequate explanation for this phenomenon is still being sought.

Analysis of the urinary recovery of CCNU (50 mg/kg) as carbamoylated conjugates in rats revealed a rank order of formation for NTHC > NCCC > NFHC, with these metabolites together accounting for the elimination of 14.3 ± 2.9 % of the dose in 24 h (Figure 51). The collective formation of 76 % of cyclohexylcarbamoyl NAC conjugates as hydroxylated analogues, although speaking for considerable phase I metabolism of CCNU prior to decomposition, reveals that substantial carbamoylating activity is retained in the form of the parent drug.

These results are consistent with what is known about the metabolism of CCNU both in vitro and in vivo (Reed and May, 1975). In vitro, CCNU undergoes rapid and extensive microsomal oxidation to afford a mixture of hydroxylated analogues (Reed, 1981). Of these, trans-3-hydroxy and cis-4-hydroxy CCNU constitute major metabolites, together accounting for 60 % (May et al., 1975) to 85 % (Hilton and Walker, 1975b) of hydroxylated CCNU metabolites. Cis-3-hydroxy, trans-4-hydroxy and trans-2-hydroxy CCNU constitute minor hydroxylated metabolites of CCNU (Hilton and Walker, 1975b; May et al., 1975). Subsequent to monooxygenation, CCNU metabolites decompose to the corresponding isocyanates, a process shown to be independent of microsomal activity (Kramer, 1989).

The monooxygenation of CCNU has been demonstrated in vivo with the identification of hydroxylated metabolites in the plasma and urine of rats. In rats, 4-hydroxy CCNU, 3-hydroxy CCNU and 2-hydroxy CCNU were identified as phase I plasma metabolites (Hilton and Walker, 1975b), and also as free urinary cyclohexylamines, ascribed to the hydrolysis of
isocyanates derived from CCNU and its hydroxylated derivatives (Kohlhepp et al., 1981). Urinary cyclohexylamines in the latter study appeared in rank order cis-4-hydroxycyclohexylamine, cyclohexylamine > cis-3-hydroxy > trans-4-hydroxy-, trans-3-hydroxycyclohexylamine. The differences in the analytical methods used by these workers and ourselves in assessing the in vivo carbamoylating activity of CCNU allow only cursory comparison of the results to be made. Thus, while the formation of NCCC in our studies parallels their finding of substantial carbamoylating activity due to CCNU, levels of urinary NFHC reported here appear to be at variance with the activity they attribute to the 4-hydroxy CCNU isomers.

Also drawn from the study by Kohlhepp et al. (1981) was the fact that alkaline treatment of urine resulted in a five-fold increase of cyclohexylamines recovered from the hydrolysis of carbamoylated peptides. Four peptide conjugates with approximate molecular weights 243, 329, 413 and 629 a.m.u. were detected, and although not identified, cysteine was found to rank highly as a constituent amino acid. It is possible that the carbamoyl NAC conjugates reported here could account for the lower molecular weight species described by these workers.

Studies on the disposition of ring-labelled [14C]-CCNU in rodents revealed that the carbamoylating species was extensively excreted in bile and urine and minimally so in feces, suggestive of the occurrence of extensive enterohepatic cycling (Oliverio et al., 1970). Considered altogether, our results and those in the literature reviewed here are consistent with CCNU undergoing hydroxylation to the 3-hydroxy and 4-hydroxy isomers, which along with the parent drug decomposes to isocyanates. The isocyanates, in turn, are trapped by GSH in the form of conjugates which are excreted in bile, and subsequently undergo enterohepatic cycling (see Discussion/Section 3.2) and/or metabolism via the mercapturic acid pathway (Tate, 1980) to the NAC conjugates which are excreted in urine (Scheme 18).

In the case of human metabolism of CCNU, SRM LC/MS allowed NCCC, previously undetected by APCI LC/MS experiments, to be also identified as a metabolite along with
NFHC and NTHC (Figure 22). For one patient in whom NAC conjugates were quantitated, the profile appeared different from that of the rat on two counts. Firstly, only one chromatographic peak was detected for the NTHC diastereomers; and secondly, the formation of the NFHC was proportionately higher, accounting for 42% of carbamoylated NAC conjugates, as opposed to 11% in rats. A larger sampling of human urine samples needs to be examined to determine whether this is reflective of a general trend. Interestingly, the metabolite profile which we observed in humans differs from an earlier account (Hilton and Walker, 1975a), and although the latter benefits from a larger patient population, there can be some validation of our observations. Whereas NFHC, NTHC and NCCC were identified as metabolites in our study, Hilton and Walker (1975a) reported only the cis and trans-isomers of 4-hydroxy CCNU as plasma metabolites. A possible reason for this discrepancy is that the latter study employed intravenous administration of CCNU, whereas in our study the drug was administered orally, a route by which compounds are likely to be subject to more extensive metabolism due to first pass effects (Pang, 1983).

It is apparent that GSH plays a key role in the conjugation of reactive species derived from the carbamoylating (this report; Stahl et al., 1987) or alkylating activity (Kramer, 1989) of CCNU. The challenge that this metabolic route presents to the reduced status of the cell could have some bearing on toxicities associated with CCNU. This issue is discussed further with respect to BCNU (Discussion/Section 2.2). In addition, GSH conjugation of isocyanates is an equilibrium process and therefore not necessarily a detoxification mechanism (Baillie and Slatter, 1991). Indeed, thiocarbamate conjugates can act as latent forms of carbamoylating activity (see Discussion/Section 5).

Previous HPLC methods for assessing the carbamoylating activity of nitrosoureas using u.v. (Brubaker et al., 1986) and fluorescence (Stahl et al., 1987) detection provide limited structural information on the isocyanate species. This report demonstrates the synergism derived from the interfacing of HPLC and MS. In addition to acquiring chromatographic information, MS probes the structural features of the analyte with respect to
molecular weight and fragmentation properties. Furthermore, SRM provides a sensitive and specific method of detection.

Figure 51. Metabolism of CCNU (50 mg/kg) in rats to carbamoylated NAC conjugates in urine over a 24 h period. Values are expressed as the mean urinary recovery ± standard error for four rats. The term "Other" refers to parent drug, covalently bound species, and metabolites not accounted for by urinary carbamoylated NAC conjugates.
Scheme 18. Proposed formation of carbamoylated thiol conjugates as metabolites of CCNU in vivo.

2.2. Metabolism of BCNU in rats and in humans

The metabolism of BCNU in rats and in humans was studied in parallel with CCNU to provide further evidence for the in vivo formation of isocyanates from nitrosoureas. Particularly important for this nitrosourea is its carbamoylating activity toward alveolar type II cells (Smith et al., 1986; Jenkinson et al., 1994) which has been associated with insidious
pulmonary fibrosis (Smith, 1989). This condition is widespread in the patient population and is major cause for concern in BCNU therapy (Weiss et al., 1981; O'Driscoll et al., 1990).

Unlike CCNU which affords a host of carbamoylating entities, BCNU decomposes to only one, CEIC. Evidence for the formation of this species in rats was borne out in the identification of SCEG and NCEC as biliary and urinary metabolites, respectively (Figure 24-27). The formation of SCEG and NCEC as metabolites in rats dosed with CEIC as the parent compound supports the contention that the isocyanate is formed as a decomposition intermediate of BCNU in vivo; however, as pointed out by Davis et al. (1993), the possibility that the carbamoylated conjugate could be formed by a catalyzed attack of GSH on the intact nitrosourea, although less likely, cannot be excluded. Our finding of SCEG and NCEC as metabolites of BCNU was recently corroborated by others (Davis et al., 1993). These workers quantitated the formation of NCEC as a urinary metabolite and a parallel was drawn in this aspect of their study and our work on CCNU. The amount of NCEC excreted by BCNU-dosed rats into urine over 24 h was 18.1 ± 3.3 % of the administered dose, which is in accordance with 14.3 ± 2.9 % of the administered dose of CCNU being excreted as carbamoyl NAC conjugates over the same duration.

Akin to the metabolism and disposition of CCNU described above (Oliverio et al., 1970), [14C]BCNU also appears to undergo extensive enterohepatic cycling in mice (De Vita et al., 1967). Supporting lines of evidence include extensive elimination of the 14C-label in urine compared to feces, and relatively high, short-term label distribution in the small intestine. It should be noted that these results were drawn from the detection of 14C-label derived from BCNU labelled in both 2-chloroethyl arms, and so conclusions were based on a summation of both carbamoylation and alkylation derived metabolites (see Introduction/Section 2.1 for chemistry). However, supporting evidence suggests that metabolites derived from both the carbamoylating (this report; Davis et al., 1993) and alkylating (Reed, 1981; Reed and May, 1975; Brakenhoff et al., 1994) activity of nitrosoureas arc substantially excrated in the urine.
The GSH dependence of BCNU metabolism, either through carbamoylation (this report; Davis et al., 1993) or GST-catalyzed alkylation (Hill, 1976; Smith et al., 1989; Weber and Waxman, 1993) possibly accounts for the depletion of hepatic GSH elicited by this drug (McConnell et al., 1979). Compared to BCNU (and CCNU), not all nitrosoureas are capable of carbamoylating GSH. For example, 1-(2-hydroxyethyl)-1-nitrosourea and chlorozotocin, because of their ability to intramolecularly form oxazolidinones, exhibit a diminished propensity to carbamoylate GSH (Stahl et al., 1987). Although not predisposed to intramolecular carbamoylation, the two nitrosoureas tauromustine and fotemustine display interesting properties toward GSH. In the presence of microsomal and cytosolic fractions, tauromustine displayed no indication of GSH conjugation (Seidegard et al., 1990). Fotemustine, on the other hand, reacted readily with GSH and NAC in vitro to form relatively stable S-linked isocyanate conjugates (Brakenhoff et al., 1994). Interestingly, the carbamoylated NAC conjugate could not be detected among the urinary metabolites of this drug, although its prior existence was inferred from the identification of urinary NAC as a putative decomposition product (Brakenhoff et al., 1993). Biliary disposition of the drug was not examined by these workers, and so the fate of a potential GSH conjugate is unknown.

The superiority of BCNU over fotemustine to carbamoylate GSH in vivo (Brakenhoff et al., 1993) and to inhibit GR (Boutin et al., 1989) could have some bearing on the markedly higher toxicity of BCNU (Laquerriere et al., 1989). It is noteworthy that the administration of the ethyl ester of GSH protected rats from BCNU toxicity (Teicher et al., 1988), the ester probably conferring its effect through the maintenance of intracellular GSH which is crucial to cytoprotection (Reed, 1990).

Screening of urine of patients on BCNU infusion therapy allowed NCEC to be identified as a metabolite in concentrations varying from 5.0 to 13.6 nmol/mL (Table 7). Urine was obtained as a single collection during the mid-point of infusion, and so the recovery of the dose as NCEC could not be determined. Because, quantitation of NCEC was carried
out solely to gain insight into the urinary levels of this metabolite, concentrations were not normalized.

It is noteworthy, that several of the drugs used in cancer polytherapy carry the potential for interaction with BCNU (Table 7). Two of the drugs used in polytherapy, namely phenytoin (Jin et al., 1991) and carbamazepine (Panesar, 1993), elevate the expression of rat hepatic P450 2B1/2B2 which is involved in the denitrosative inactivation of BCNU (Weber and Waxman, 1993). Consistent with this finding, studies on the antitumor effect of BCNU in rats revealed that this activity was antagonized by PB treatment (Levin et al., 1979). Surprisingly, however, the antitumor activity of BCNU was unaffected by the P450 2B1/2B2 inducer phenytoin. A parallel situation was also obtained in the case of dexamethasone. Whereas dexamethasone induction elevated BCNU denitrosation in vitro (suggestive of P450 3A1/3A2 involvement) (Weber and Waxman, 1993), this agent did not oppose the antitumor effect of BCNU in rats (Levin et al., 1979).

The paucity of literature about the influence of P450 inducers on the metabolism and efficacy BCNU, compounded by the apparent inconsistencies in the findings alluded to above, make the pattern of interaction between these agents (i.e. phenytoin, carbamazepine, dexamethasone) and BCNU in humans unpredictable at this time. Furthermore, unlike the rat, P450 2B1/2B2 and 3A1/3A2 are not orthologously expressed in humans (Boobis et al., 1990), P450 3A3/3A4 being instead the most responsive form to PB-like inducers (phenytoin and carbamazepine) and dexamethasone (Wrighton and Stevens, 1992; Wrighton et al., 1993). The complexity of the processes which modulate the therapeutic index of nitrosoureas underscore the need for methods to evaluate the formation of isocyanates in vivo. In this respect, the detection of urinary NAC conjugates described here could fill a role for providing a non-invasive probe to monitor the carbamoylating activity of nitrosoureas in patients on chemotherapy.
3. Metabolism of N-formyl amphetamine in rats

3.1. Mass spectrometry

The metabolism of NFA in rats was examined with a view to characterizing the chemical species that arise as a consequence of in vivo carbamoylation. For this purpose NFA, deuterated in the aromatic ring, was synthesized for administration to rats in order to provide information on the carbamoylating element of NFA when traced by LC/MS and LC/MS/MS. \(^{2H_5}\)NFA was considered to be an ideal candidate as a stable isotope labelled analogue because aromatic protons are chemically inert under physiological conditions (March, 1985a), and biotransformations involving aromatic proton removal are subject to modest isotope effects (Trager, 1980; Ortiz de Montellano, 1986) and consequently minimal metabolic shifting.

The first step in the profiling strategy as applied to bile, involved the recording of \(Q_1\) scan data as a contour using SCIEX MacSpec3.2 software (Figure 30). By this approach a summed mass spectrum (y-axis) was resolved along a chromatographic time axis. The advantage of this technique resides in the fact that chromatographic and mass spectral data are simultaneously recorded, which allows separated metabolites to be more readily discerned in their y-dimension TIC mass spectra (Rudewicz et al., 1993). In a previously reported isotope-cluster study, the entire duration of the chromatographic run was recorded as a single, summed, background-subtracted, mass spectrum to decipher metabolite-related doublets which varied dramatically in ion intensity (Weidolf and Covey, 1992). Isotope clusters virtually sequestered in the TIC using that approach could probably be more readily unmasked with the chromatographic resolution of the contour output.

Distinctly apparent in the display of LC/MS data as a contour was the fact that doublets were not completely symmetrical but skewed to the lower molecular weight with increasing retention time (Figure 30). Deutero analogues preceded their protio partners by 0.04 - 0.07 min, consistent with the higher polarity displayed by the former under reverse phase HPLC conditions (Tanaka and Thornton, 1976; Honma et al., 1987). One of the
inherent limitations of this approach is the uncorrected registration of background interference as was apparent at m/z 390 - 394 and m/z 398 - 402 (Figure 30). Nonetheless, once doublets were identified in the contour, subtraction of background elements at time intervals before and after the doublet allowed a useful summed mass spectrum to be reconstructed if necessary.

Two modifications to improve this approach to metabolic profiling are apparent. Firstly, the scanning of a narrower Q1 mass range in repeat experiments would improve ion statistics by affording an increased dwell time per ion with a concomitant increase in sensitivity. Secondly, a less intensive gradient increase of the HPLC organic modifier would afford an improved separation of metabolites and concomitantly facilitate the discernment of isotopic doublets. It is possible that these developments could uncover the presence of metabolites which because of their low concentrations are masked under the present method.

This report, as well as previous studies (Baillie et al., 1989; Weidolf and Covey, 1992; Lanting et al., 1993), serve to underscore the synergistic utility of LC/MS, LC/MS/MS and stable isotope methodology in the characterization of metabolite entities. In the work here, SMPG was confirmed as a biliary metabolite of NFA (Mutlib et al., 1991), and serendipitously, MPIC derivatives of cysteinylglycine, cysteine and NAC were identified as novel metabolites in bile. Only the NAC conjugate was detected in urine.

3.2. Excretion of mercapturate pathway metabolites

The formation of SMPG as a biliary metabolite was thought to be a consequence of NFA bioactivation to MPIC which reacted with hepatic GSH to afford the conjugate (Scheme 19). However, the additional finding of carbamoylated cysteinylglycine, cysteine and NAC conjugates as metabolites in rat bile was very interesting. In the usual course of the mercapturic acid cascade, GSH and NAC conjugates are preferentially excreted in bile and urine, respectively (Scheme 18), because of their physico-chemical properties (Levine, 1978). This principle holds true for carbamoylated GSH and NAC conjugates as excretory products of nitrosoureas (vide supra) and formamides (Threadgill et al., 1987; Mraz et al., 1989;
Mutlib et al., 1990; 1991). The biosynthesis and disposition of mercapturic acids is, for the most part, explained by the hepatorenal coordinated model proposed by Inoue et al., (1982; 1984. According to this model, GSH conjugates formed in the liver, where the activities of P450-mediated xenobiotic bioactivation and GSH conjugation are relatively high (Stevens and Wallin, 1990), are transported by the circulatory system to the kidney where the sequential activity of γ-glutamyltranspeptidase (γ-GT) and non-specific peptidases continue the cascade to the cysteinyl conjugate (Tate, 1980). The cysteinyl conjugate in turn, is either N-acetylated in the kidney or returned via the circulatory system to the liver for this biotransformation to occur before final renal elimination (Inoue et al., 1982; 1984).

The construct of this model does not account for the presence of all the mercapturic acid pathway conjugates of MPIC found as biliary metabolites of NFA. A review of the literature revealed that the biliary excretion of GSH, cysteinylglycine, cysteine and NAC conjugates observed here for NFA was an uncommon phenomenon, and has been reported for the in vivo metabolism of very few compounds including pentachlorothioanisole (Bakke et al., 1990), 1,2,4-trichlorobenzene (Bakke et al., 1992), and benzyl chloride (Caldwell et al., 1989). The question inevitably arises as to what factors govern this pattern of metabolism and biliary excretion.

The design of this study with respect to the times of dosing and the collection of bile, undoubtedly invited participation from renal and enteric systems in the ultimate biliary elimination of mercapturate pathway metabolites. The collection of bile after two doses of NFA - one at 24 h before bile duct cannulation and another immediately thereafter, allow for extensive metabolism of MPIC conjugates by hepatorenal cycling (Inoue et al., 1982; 1984) (vide supra), as well as enterohepatic cycling (Stevens and Wallin, 1990). With respect to enterohepatic cycling, it is conceivable that the γ-GT, peptidase and N-acetyl transferase (NAT) activity present in the intestinal mucosa account for substantial conversion of SMPG to its secondary metabolites which could be transported back to the liver for further mercapturate pathway metabolism upon their second hepatic transit (Monks et al., 1990;
Stevens and Wallin, 1990). It is interesting that the liver also possesses significant N-deacetylase activity (Commandeur et al., 1991) which, by opposing NAT, conceivably modulates the cysteine/NAC conjugate ratio which finally emerges upon biliary elimination.

In a recent review Hinchman and Ballatori (1994) address the concept of the liver assuming a dominant role in the mercapturate pathway without recruitment of the kidney. When these workers infused 0.3 μmol and 3.0 μmol of 1-chloro-2,4-dinitrobenzene (CDNB) into isolated rat and guinea pig liver it was found that GSH, cysteinylglycine, cysteine and NAC conjugates were all excreted in the bile of both species (Hinchman et al., 1991). NAC conjugate formation was more efficient in the guinea pig and became saturated at the higher concentration of CDNB. It was concluded that the GSH conjugate excreted into bile was metabolized intrahepatically to the cysteine conjugate which was in turn transported back into the hepatocyte across the canalicular membrane for N-acetylation and ultimate reexport as the mercapturic acid. This suggestion is certainly tenable in the light of reported γ-GT activity in hepatocyte canaliculi (Meister et al., 1976), biliary tract (Ballatori et al., 1988) and bile (Rosalki, 1975). Thus, consistent with the findings of Hinchman and Ballatori (1994), it is conceivable that the metabolite profile of NFA observed here could, in part, be the result of biliary-hepatic cycling and possibly affected by dose.

The study by Hinchman et al., (1991) brings other aspects of the metabolism of GSH conjugates into focus. Among animal species, the ratio of kidney/hepatic γ-GT activity ranks high in the rat (Hinchman and Ballatori, 1990). In this light, there is speculation that the uncommon occurrence of NAC conjugates as metabolites in the bile of rats could be reflective of the saturation of hepatic γ-GT at high doses of xenobiotics eliciting a diversion from the biliary route to an overwhelming renal contribution.

Another consideration related to the disposition of metabolites of the mercapturic acid pathway is the route of xenobiotic administration. In the study reported here, NFA was injected i.p. and consequently destined for the portal circulation (Lukas et al., 1971) and extensive first-pass metabolism (Pang, 1983). Because the bioactivation of formamides to
isocyanates takes place to a large extent in the liver (Pearson et al., 1987a; 1987b; Shaw et al., 1988; Tulip and Timbrell, 1988), the rapid sequence of events involving NFA bioactivation to MPIC, GSH conjugation to form SMPG, and the elimination of the GSH conjugate and its secondary mercapturate pathway metabolites, is quite likely to be favoured by the hepatic route.

Formamide-derived isocyanates, as a consequence of being generated in the liver, are likely to be predisposed to hepatic excretion. On this note, it is worthwhile to consider how isocyanates could themselves be metabolized and eliminated when exposure arises by extrahepatic routes. For example, inhalational exposure under environmental (Heylin, 1985) and industrial (Vandenplas et al., 1993) conditions, could conceivably result in rapid conjugation of the isocyanate to pulmonary reserves of GSH (Cantin et al., 1987). In this situation, GSH conjugates proceeding from the lung are likely to be metabolized in the kidney and excreted therefrom (as the NAC conjugates) moreso than the liver because of the higher levels of mercapturate pathway enzymes in the kidney (Tate, 1980; Hinchman and Ballatori, 1990).

The fact that thiol conjugation of isocyanates is an equilibrium process (Baillie and Slatter, 1991) could also play a role in the biliary metabolite profile of NFA. This consideration becomes apparent when it is borne in mind that GSH, cysteinylglycine, and cysteine are excreted in the bile of rats at rates of 100, 50 and 20 nmol/min/kg, respectively (Madhu et al., 1993). It is conceivable that these free thiols and mercapturate pathway conjugates, at the alkaline pH of bile (Levine, 1978), could be involved in an equilibrium exchange of the isocyanate species, thereby non-specifically altering the thiol metabolite profile upon biliary transit.

Screening of the urine of NFA-dosed rats revealed detectable levels of only NMPC in accordance with what is known about the excretion of thiol conjugates by this route. Because of their high molecular weight SMPG and SMPCG are selectively favoured by the biliary route (Levine, 1978). NMPC, on the other hand, is a likely candidate for urinary excretion for
two reasons: (1) this metabolite lies at the lower molecular weight threshold for biliary excretion (Levine, 1978), and (2) mercapturic acids are good substrates for the organic anion transporter of renal proximal epithelial cells (Zhang and Stevens, 1989). As our identification of biliary NMPC indicates, however, it is possible that a portion of this metabolite could escape renal extraction and be transported back to the liver for excretion. The cysteine conjugate of MPIC (SMPC) was not detected as a urinary metabolite of NFA, nor was the cysteine conjugate of MIC in rats dosed with NMF (Baillie et al., 1989). This phenomenon could be illustrative of the fact that cysteine conjugates, unlike NAC conjugates, are unsuitable candidates for transport mechanisms present in the kidney (Monks et al., 1990).

In summary, these findings illustrate the utility of LC/MS contour formatting, LC/MS/MS and isotope cluster methodology in metabolism studies by deciphering a novel pathway in the biotransformation and disposition of NFA. Whether this pattern is unique to NFA, or is characteristic of formamides as a class of xenobiotics needs to be addressed. The fact that NMPC excretion partitions between both bile and urine indicates that, for some compounds, alternative pathways of disposition exist for NAC conjugates. This phenomenon is germane to the non-invasive analysis of urinary mercapturates as an index of the primary event of GSH conjugation with a reactive chemical species (Vermeulen, 1989); (Mraz et al., 1991). It may be necessary to give consideration to the fact that mercapturate pathway metabolism could be modulated by the dose of the xenobiotic, animal species, route of xenobiotic exposure (or administration) and the type of thiol conjugate formed.
Scheme 19. Proposed pathway for the metabolism of N-formyl amphetamine in rats to carbamoylated GSH, cysteinylglycine, cysteine and NAC conjugates.
4. In vitro metabolism of N-formyl amphetamine by hepatic subcellular fractions

4.1. Microsomal metabolism of NFA compared to model P450 catalytic activities

In vitro metabolism of NFA to MPIC

Although the microsomal metabolism of NMF and DMF has received considerable attention (Cross et al., 1990; Hyland et al., 1992; Gescher, 1993), similar studies for high molecular weight formamides were absent from the literature. Investigation of the latter compounds was considered necessary to determine the enzymes and mechanisms involved in their metabolism. Our research in this area was approached by using the bioactivation of NFA to its reactive product MPIC as a model reaction. The metabolic formation of unstable chemical species presents a complication when it is desirable to characterize and quantitate the product. One of the strategies used to solve this problem is to conjugate the reactive product to a target molecule and thereby generate a relatively stable, quantifiable adduct. Molecules serving this purpose include adenosine, albumin and GSH (Guengerich et al., 1991). GSH plays a role in the trapping of MIC as the thiocarbamate conjugate SMG during the microsomal bioactivation of NMF (Cross et al., 1990; Hyland et al., 1992). In these studies, SMG was treated with alkaline ethanol to afford ethyl N-methylcarbamate which was analyzed by GC. One of the primary complications noted in this approach is that the conversion of SMG to the ethyl carbamate ester is not quantitative due to the decomposition of SMG incurred under basic conditions (Mraz, 1988). In the approach used here, the GSH conjugate was analyzed as such, thereby circumventing any such loss of product under base treatment.

SMPG, formed by the trapping of MPIC with GSH, was stable under the acidic conditions generated upon termination of the microsomal reaction with TFA. Furthermore, LC/MS/MS analysis using the SRM of the transition m/z 469 (MH+)/340 (Y2) afforded a specific assay for the product with a linear response ($r^2 = 0.992$) over the concentration range for the microsomal product (Figure 38).

In our attempt to establish the involvement of inducible microsomal and mitochondrial P450 isoforms in the catalysis of NFA metabolism, PB and acetone were used as agents for
the treatment of rats. Values obtained for total P450 content of hepatic microsomes from untreated and PB treated rats (Table 8) were comparable to previously reported values (Niranjan et al., 1984). The elevation of total microsomal P450 content with PB treatment was in accordance with the capacity for this agent to stimulate proliferation of the endoplasmic reticulum (Waxman and Azaroff, 1992). Acetone treatment elicited marginal elevation in total P450 content.

The desaturation of NFA to MPIC was supported by microsomes and NADPH (Table 9). Although these criteria point to the involvement of P450 catalysis, it should be noted that microsomal NADPH-P450 reductase, which utilizes NADPH as a cofactor, is a metabolizing enzyme in its own right (Backes, 1993). Metyrapone, a reputedly broad spectrum inhibitor of P450 (Ortiz de Montellano and Reich, 1986), has been previously used to characterize the involvement of this group of enzymes (Kaminsky et al., 1992; Mani et al., 1993). This inhibitor elicited a 63 % reduction in the catalysis of NFA desaturation. Although this result invites the possibility that microsomal enzymes other than P450 could be involved in NFA desaturation, e.g. NADPH-P450 reductase, it does not eliminate the likelihood of obligate P450 catalysis. Evidence indicates that metyrapone is not entirely a broad-spectrum P450 inhibitor. Consistent with this argument is the fact that the extent of inhibition of microsomal P450 activity by metyrapone is modulated by inducing agents (Goeptar et al., 1993; Kedderis et al., 1993), which suggests selectivity in isozymic inhibition. Support for this contention can be viewed in the metabolism of lovastatin, in which the inhibition of three positional oxidations by metyrapone, ascribed to the catalysis of different isoforms, was exerted to the extent of 48, 75 and 100 % (Vyas et al., 1990). In other studies, P450 catalysis was unaffected by metyrapone (Tunek and Oesch, 1979; Yang et al., 1990; Renaud et al., 1993).

The microsomal desaturation of NFA proceeded at 1.22 nmol/min/nmol-P450, which was higher than the reported value for the conversion of NMF to MIC in rat liver microsomes (0.44 nmol/nmol-P450/min) (Hyland et al., 1992). Interestingly, a higher rate of formamide desaturation was also observed in microsomes from mouse liver for the larger substrate N-
ethylformamide (NEF) (1.26 nmol/min/nmol-P450) over NMF (0.42 nmol/min/nmol-P450) (Cross et al., 1990). The difference in catalytic parameters for NMF, NEF and NFA could have some bearing on the P450 isozymes involved in their catalysis; however, the absence of available information outside of the studies mentioned here preclude any further evaluation of these findings.

Metabolism of NFA by microsomes obtained from acetone and PB treated rats presented a profile unlike NMF. Whereas the microsomal metabolism of NMF to MIC was elevated to 240 % of control in acetone-treated rats (Hyland et al., 1992) and decreased to 67 % of control in PB-treated mice (Cross et al., 1990), the level of metabolism of NFA to MPIC was unaffected by acetone, and modestly elevated to 125 % of control with PB treatment (Figure 41). These data do not describe a prominent role for either P450 2E1 or 2B1/2B2 in NFA bioactivation. On the other hand, P450 2E1 is the major catalytic isoform in the desaturation of NMF (Hyland et al., 1992). Should P450 2E1 also contribute to some extent in the desaturation of NFA, it is possible that this influence could be masked with the relatively high substrate concentration of 5 mM used here. Such a scenario can be found in the microsomal demethylation of N,N-dimethylnitrosamine (DMN) (Yang et al., 1990). P450 2E1 is distinguished by its low K_m for DMN, the involvement of other isoforms becoming apparent at high substrate concentrations.

An attempt was made to characterize the involvement of P450 2E1 and 2B1/2B2 using the mechanism based inhibitors DEDTC (Guengerich et al., 1991) and orphenadrine (Murray and Reidy, 1990), respectively. The rationale behind the inhibitor concentrations used in these studies needs to be addressed. Orphenadrine at 25 μM was within the range of concentrations used by Reidy and coworkers (1989) in their plotting of the competitive inhibition of PROD activity (P450 2B1/2B2-selective). DEDTC at 500 μM was derived from a study by Guengerich and associates (1991) as the concentration required to inhibit the P450 2E1-selective biotransformation of the substrates chlorzoxazone and DMN by ca 88 % and 72 %, respectively. Consistent with the isozymes induced by PB (P450 2B1/2B2) and acetone
(P450 2E1), experiments involving the inhibition of NFA metabolism with DEDTC (for P450 2E1) and orphenadrine (for P450 2B1/2B2) were not performed in microsomes prepared from PB and acetone-treated rats, respectively. The marginal inhibition exerted by DEDTC and orphenadrine in microsomal incubations, coupled with the weak influence of acetone and PB induction (vide supra), speaks against an active involvement by P450 2E1 or 2B1/2B2 in NFA desaturation. Interestingly, DEDTC elicited its most profound inhibition of NFA desaturation in microsomes obtained, not from acetone treated rats, but from controls. This finding suggests that acetone apparently suppresses hepatic microsomal P450 isoforms more responsive to the DEDTC inhibition of this biotransformation. This phenomenon was not characteristic of microsomal PNH activity in which case acetone induction, as expected, amplified the inhibition of DEDTC (vide infra). Further experiments on formamide desaturation need to be performed to rationalize these findings.

**PNH and PROD as model biotransformations for microsomal P450 2E1 and 2B1/2B2**

In order to view the microsomal metabolism of NFA in perspective with model biotransformations catalyzed by P450 2E1 and 2B1/2B2, the activities of PNH and PROD, respectively, were investigated. Subsequent to a report of its induction by ethanol (Reinke and Moyer, 1985), p-nitrophenyl hydroxylation was considered to be a marker for P450 2E1 and was developed for this purpose (Koop, 1986). Although not as selective as DMN N-demethylation, PNH ranks highly as a facile assay for P450 2E1 activity (Lucas et al., 1990; Tassaneeyakul et al., 1993). Our value for microsomal PNH activity from untreated rats, 0.47 nmol/min/nmol-P450 (0.57 nmol/min/mg-protein), was consistent with the lower end (0.61 nmol/min/mg-protein, Surbrook and Olson, 1992) of values reported in the literature (Lucas et al., 1990; Hyland et al., 1992). Acetone, in accordance with its activity as an inducer of P450 2E1 (Koop et al., 1985; Johansson et al., 1988), elicited an elevation in PNH activity to 200% of control in our hands, an induction within the reported range of 176% (Forkert et
PB exerted no significant influence on PNH activity, lending support to the use of this reaction as a marker for P450 2E1.

Subsequent to preliminary reports of DEDTC as a selective inhibitor of P450 2E1 (Brady et al., 1991; Guengerich et al., 1991), the impairment of microsomal catalysis by this agent has been utilized to delineate the involvement of this isoform (Kharasch and Thummel, 1993; Urban et al., 1994). Inhibition of PNH activity by DEDTC was most pronounced (96 %) in microsomes from acetone treated rats. This phenomenon, which has been observed by others (Surbrook and Olson, 1992), was considered reflective of an acetone-mediated shift in microsomal P450 composition to an isoform more responsive to DEDTC inhibition.

Microsomal PROD has been characterized as a highly selective marker for P450 2B1/2B2, exhibiting some 283-fold increase in activity upon PB induction (Burke et al., 1985). PB induction of PROD, although not as dramatic in this study, was clearly manifest in a 19-fold elevation in activity over control (Figure 40 C). Also apparent was an 8-fold induction of this marker by acetone, a pattern which has been observed by others (Winters and Cederbaum, 1992). These results attest to the fact that, in addition to P450 2E1, acetone also induces P450 2B1/2B2 (Johansson et al., 1988).

In the event of its preincubation with microsomes, orphenadrine is a selective noncompetitive inhibitor of P450 2B1 and 2B2, although it is thought that some partiality to the former isoform exists (Reidy et al., 1989). The selectivity of this inhibitor for P450 2B1/2B2 was aptly borne out in the complementary findings of its dramatic reduction of microsomal PROD and its failure to perturb PNH activity. In this respect, orphenadrine stood apart from the less selective DEDTC which elicited ca. 75 % reduction in PROD activity in microsomes from acetone and PB-treated rats. As a result, we were prompted to investigate the effect of DEDTC concentration on microsomal PROD activity. PROD catalysis by microsomes from rats treated with either acetone or PB was lowered by 32 % even at 100 μM DEDTC (Figure 41), a concentration at which P450 2E1-selective metabolism of chlorzoxazone and DMN was reduced by ca 72 % and 60 %, respectively (Guengerich et al.,
In a recent report, the specificity of DEDTC as an inhibitor of P450 2E1 was challenged, based on its inhibitory action toward the microsomal activity of animals treated with inducers of isoforms other than P450 2E1 (Kedderis et al., 1993). These results together suggest that although inhibition by DEDTC could indicate the involvement of P450 2E1 catalysis by microsomes, the participation of other isoforms cannot be excluded solely on this basis.

Microsomal metabolism of the high molecular weight formamide NFA carried no resemblance to either PNH or PROD model reactions, arguing against a singular involvement of either P450 2E1 or 2B1/2B2 in NFA bioactivation. The evidence here suggests that although N-formyl metabolism depends on the presentation of the formamide function to the catalytic site of P450, isozymic involvement in this process could be controlled by the N-formyl substituent. It has been suggested that P450 2E1 acts as a "molecular sieve" in its catalysis, being partial to low molecular weight substrates (Guengerich et al., 1991) like NMF (Hyland et al., 1992). Our results could be a reflection of this hypothesis. Alternatively, as suggested above, P450 2E1 activity could be covered by the participation of other isoforms with the relatively high substrate concentration used in these studies.

4.2. Microsomal P450-mediated formyl metabolism: A mechanistic perspective

The microsomal metabolism of NFA to MPIC reported here casts P450 as a desaturase, an uncommon role for this group of enzymes (Guengerich and MacDonald, 1990; Guengerich, 1993). Subsequent to the characterization of this catalytic pathway for lindane (Chadwick et al., 1975), few reports for other substrates have ensued (Nagata et al., 1986; 411(Rettie et al., 1987); 1601(Vyas et al., 1990); 1602(Vickers et al., 1990) (Figure 52). In the case of formamide oxidation, the isocyanate product is unusual in that it possesses a cumulated unsaturated system. Speculation on the mechanism for P450-mediated oxidation of secondary formamides has revolved around the primary deuterium isotope effect exhibited by CH$_3$NHC$^2$HO ($^2$H]NMF) during the course of its in vivo metabolism (Threadgill et al.,
Formation of the metabolites SMG ($k_H/k_D = 7.0$), AMCC ($k_H/k_D = 4.5$) and methylamine ($k_H/k_D = 5.5$) strongly implicates formyl C-H cleavage as the rate-determining step in the genesis of the isocyanate (Guengerich, 1992; Gescher, 1993); however, no solid mechanistic explanation of the catalytic process has been advanced. The following mechanism is proposed for NFA, based on what is known of the kinetic deuterium isotope effect with [$^2$H]NMF (Threadgill et al., 1987) and the desaturation of ethyl carbamate (Guengerich and Kim, 1991) (Scheme 20). In essence, (1) preliminary abstraction of a formyl hydrogen atom from either (A) carbon or (B) nitrogen gives rise to the corresponding free radical which could undergo either (2) a subsequent hydrogen atom abstraction (from the position vicinal to the radical) to afford the isocyanate MPIC and H$_2$O, or (3) recombination with the ferryl oxy radical to produce (A) the carbamic acid (carbon hydroxylation) or (B) the hydroxamic acid (nitrogen hydroxylation). The carbamic acid, because of its unstable nature, decomposes to amphetamine with the concomitant loss of CO$_2$. (4) It is also possible that P450 could be involved in hydroxyl radical-mediated catalysis (vide infra).

Because the isocyanate (Pathway 2) does not disclose the identity of its (carbon/nitrogen) radical precursor (Pathway 1), evidence for the putative intermediate is borne out in the downstream products of radical recombination (Pathway 3). Evidence in support of an initiating formyl C-H cleavage (Pathway 1A) can be viewed in Pathway 3A through the metabolites methylamine from NMF (Threadgill et al., 1987; Tulip and Timbrell, 1988) and $^{14}$CO$_2$ from $[^{14}$C]formyl-NMF (Kestell et al., 1985). It should be noted in mitigation, however, that the metabolism of the formamide to the amine does not provide conclusive evidence for a carbon-centred radical intermediate in vivo, because it is possible that the amine could also arise through the hydrolysis of the isocyanate. Whereas this process cannot be controlled in vivo, the presence of supplemented GSH to trap the isocyanate as a conjugate in microsomal incubations minimizes the likelihood of (isocyanate) hydrolysis in vitro. The development of an assay to identify the formation of amphetamine as a microsomal product revealed that even the mild alkaline conditions used to extract the putative
amphetamine metabolite from incubations, resulted in substantial hydrolysis of the GSH conjugate to form amphetamine (Figure 42). An alternative strategy to characterize amphetamine as a metabolite would involve treatment of the microsomal reaction with concentrated strong base, so as to optimally convert SMPG to amphetamine. The amount of amphetamine detected can then be ascribed to a total from SMPG decomposition and the metabolite amphetamine. The latter can be calculated on a mole basis as the net value of SMPG from total amphetamine i.e. amphetamine_{metabolite} = amphetamine_{total} - SMPG. The formation of [\textsuperscript{2}H\textsubscript{5}]amphetamine from the internal standard [\textsuperscript{2}H\textsubscript{5}]SMPG will correct for the recovery of primary amine arising from the decomposition of the GSH conjugate.

Although the possibility of formamide metabolism to a hydroxamic acid has been considered (Tulip and Timbrell, 1988; Gescher, 1993), review of the literature has revealed neither the design of a targeted search for this putative metabolite nor its identification. The concept of selective hydrogen atom abstraction giving rise to different products, alluded to in Scheme 20, has been reviewed for acetaminophen (Vermeulen et al., 1992). This aspect of formamide metabolism remains open to future study.

Should P450-mediated formyl metabolism proceed through a radical intermediate, what are the factors which govern the partition between desaturation and hydroxylation? Baillie and associates have proposed that the substrate, by virtue of its electronic properties, could facilitate the desaturation process as borne out in the elevated partition ratio (olefin:alcohol) of 2-propyl-2-pentenoic acid (2-ene VPA) to 2-propyl-2,4-pentadienoic acid (0.45) (Kassahun and Baillie, 1993) compared with VPA to 2-propyl-4-pentenoic (0.015) (Rettie et al., 1987). It was thought that resonance stabilization of the allylic radical from 2-ene VPA increased its longevity at the catalytic site with a concomitant propensity for desaturation to the conjugated diene. One oversight of this hypothesis is that it fails to consider the P450 isoform involved in the catalytic process, and inferentially the imposed orientation of the substrate at the active site. It was recently demonstrated that the differential
presentation of acetaminophen to heme iron in P450 1A1 and P450 2B1 led to preferential quinone formation (P450 1A1) over 3'-hydroxylation (P450 2B1) (Myers et al., 1994).

Another fact to be considered in this vein, is that different P450 isoforms are involved in the catalysis of substrates which are metabolized through a radical intermediate as described for NFA in Scheme 20 e.g. ethyl carbamate - P450 2E1 (Guengerich and Kim, 1991), 2-ene VPA - P450 2B1/2B2 (Panesar, 1993) and testosterone - P450 3A1/3A2 (Nagata et al., 1990) (Figure 52). Furthermore, it was demonstrated that although P450 3A was the major contributor to the hydroxylation of lovastatin other isoforms were involved in the desaturation to the exocyclic olefin (Wang et al., 1991). Taken together, these considerations strongly suggest that the partition between desaturation and hydroxylation could be governed by the catalytic P450 isoforms.

Earlier studies in our laboratory (Mutlib and Abbott, unpublished observations) demonstrated that NFA could be chemically converted to MPIC with Fe^{2+}/H_{2}O_{2} (Fenton's reagent). In like fashion, Fenton chemistry is obtained in biological systems (Rashba-Step et al., 1993). During the course of P450 catalysis H_{2}O_{2}, formed as a result of either (1) a one-electron reduction of prosthetic heme, (2) superoxide release from NADPH-P450-reductase or (3) two-electron reduction of dioxygen, is released upon uncoupling of mixed function oxidase catalysis (Kappus, 1993). In the presence of heme-containing proteins (e.g. P450), H_{2}O_{2} decomposes to HO' (Dix and Aikens, 1993) which has been implicated as an active oxidant species in some P450-mediated biotransformations (Ingelman-Sundberg and Hagbjork, 1982). We sought to investigate the involvement of such a mechanism in the metabolism of NFA (Scheme 20, Pathway 4). Catalase is a scavenger of H_{2}O_{2} (Ingelman-Sundberg and Hagbjork, 1982), and inclusion of 100 U of this enzyme in reconstituted systems inhibited (80 - 94 %) the P450 mediated oxidation of aniline (Ingelman-Sundberg and Ekstrom, 1982) and benzene (Johansson and Ingelman-Sundberg, 1983). Addition of 1000 U of catalase to microsomal incubations resulted in a modest 15 % reduction in the metabolism of NFA which appears suggestive of a minor role for hydroxyl radical catalysis by P450.
(Table 9). However, there exists rational basis for continued attention in this area. Whereas, catalase was inhibitory in reconstituted systems, other agents (e.g. catechol, DMSO) have been used as inhibitors in microsomal incubations (Johansson and Ingelman-Sundberg, 1983), perhaps indicative of the superior efficacy of organic reagents in microsomes.

It is noteworthy that among the P450 isoforms which are involved in hydroxyl radical monooxygenation, P450 2E1 is a leading candidate (Kappus, 1993). A corollary to the association of P450 2E1 with this mechanism of catalysis is that NMF desaturation is mediated essentially by this isozyme (Hyland et al., 1992). It is tempting to speculate that such a mechanism, at least in the case of NMF, could be contributory to formamide bioactivation.
Figure 52. Structures of compounds which undergo P450-mediated desaturation. Arrows indicate the position at which desaturation occurs.

Scheme 20. Proposed mechanism for the P450-mediated oxidation of NFA.
4.3. Mitochondrial metabolism of NFA

Subsequent to the finding that mitochondrial P450 was involved in the bioactivation of aflatoxin B₁ (Niranjan and Avadhani, 1980), reports in the literature have served to solidify the contention that mitochondrial P450 is involved in the metabolism of xenobiotic compounds (Honkakoski et al., 1988; Shayiq and Avadhani, 1989; 1990). Lines of evidence used to characterize P450 catalysis include: (1) metabolism in intact mitoplasts when supported by glutamate/malate which stimulates the production of NADPH in situ; and (2) NADPH-dependent catalysis by mitoplasts when sonicated to allow the access of the cosubstrate, (which is otherwise unable to access intact mitochondria when provided as a supplement) (Niranjan et al., 1984). We hypothesized that, should formamides be substrates for mitochondrial P450, then isocyanates could be generated within mitochondria.

The reactivity of isocyanates generated by mitochondrial P450 in situ, together with the critical dependence of cellular function on mitochondrial activity introduced the possibility of two immediate lines of toxicity. Firstly, mitochondrial GSH is crucial to the functioning of the cell, and its depletion often precedes cell injury (Reed, 1990). Secondly, the inactivation of mitochondrial glutathione reductase by isocyanates (Babson and Reed, 1978) could exacerbate the oxidative burden of GSH depletion. Support for the concept of intramitochondrial bioactivation of secondary formamides was viewed in the NMF-mediated perturbation of mitochondrial Ca²⁺ homeostasis in mice and in isolated mitochondrial preparations (Whitby et al., 1984).

Intact well-coupled mitochondria supplemented with glutamate/malate catalyzed the conversion of NFA to SMPG (Figure 44). Because these mitochondria were minimally purified (compared to mitoplasts), there existed ca 5 % microsomal contamination (based on total NADPH cytochrome c reductase activity); however, in the absence of NADPH, as was obtained here, microsomes formed no detectable product (Table 9). Thus, the formation of SMPG was considered solely attributable to mitochondria.
This finding presented the likelihood that mitochondrial P450, analogous to microsomal P450, could be involved in this process. We sought to test this hypothesis by investigating the NADPH-supported metabolism of NFA in purified sonicated mitoplasts. Purification measures beyond those reported by Shayiq et al. (1991) were employed to minimize microsomal contamination in mitoplasts. These included a second digitonin treatment and five washings instead of three after formation of the mitoplasts. Values for total P450 content of mitoplasts, 0.06 - 0.13 nmol/mg-protein (Table 8), were considerably lower than those reported in the literature, 0.20 - 0.50 (Niranjan et al., 1984), resulting in a low overall yield. Levels of microsomal contamination based on total NADPH-cytochrome c-reductase activity were low (≤0.21 %) (Table 10), consistent with the value reported in the literature at ca 0.9 % (Niranjan et al., 1985). However, when microsomal contamination was evaluated based on the specific activity of the marker, values of 4.6 - 6.6 % were obtained.

To more rigorously characterize the nature of the contamination we employed the sensitive, specific ECL™-coupled immunochemical detection of microsomal P450 2C11, which to our knowledge is not othologously expressed in mitochondria. This assay revealed microsomal contamination to be in the vicinity of 2.0 - 5.4 %. Although microsomal contamination was modest, the catalytic involvement of P450 from this source could not be unequivocally discounted, given the relatively low level of SMPG formation in sonicated mitoplasts supported by NADPH (Table 11). Further studies into the possible involvement of mitochondrial P450 in NFA bioactivation will involve the use of a reconstituted system with purified P450mt3 and adrenodoxin/adrenodoxin reductase which has been supplied by Drs. Avadhani and Addya.

Because we were unable to unequivocally demonstrate that mitochondrial P450 catalyzes in the formation of SMPG, the mechanism by which this occurs remains unclear. Apart from P450 catalysis, it is conceivable that intramitochondrial Fenton type processes, analogous to our observations in a chemical system (Mutlib and Abbott, unpublished observations), could mediate this transformation. This reaction would be fuelled by H_2O_2
which is generated as a consequence of aerobic respiration at ambient levels of 125 pmol/min/mg-protein in rat hepatic mitochondria (Sohal et al., 1990). Although the argument for intramitochondrial toxification remains to be solidified for formamides, this concept has been envisaged for benzene (Johansson and Ingelman-Sundberg, 1983). These workers perceived the mitochondrial bioactivation of benzene as a distinct possibility, advancing this hypothesis to account for the site-specific covalent binding of this substrate (Gill and Ahmed, 1981).

5. Toxicological significance of the reactivity of thiocarbamate conjugates

5.1. Reactivity of thiocarbamate conjugates

It is now recognized that GSH conjugation of electrophiles is not always a detoxification process, with substrate reactivity being actually amplified or delayed in the form of the conjugated species (van Bladeren, 1988). With respect to the latter scenario, it has been proposed that the nucleophilic addition of GSH to an isocyanate species temporarily sequesters its carbamoylating activity, only to be unmasked by E1cB reversal of the conjugation equilibrium under physiological conditions (Baillie and Slatter, 1991). We envisioned in this mechanism the possibility that formamide and nitrosourea-derived isocyanates formed at one physiological location, upon binding to GSH, could be transported by biological fluids (e.g. bile and blood) in the form of the conjugate to sites further afield where the isocyanate could be released upon equilibrium reversal (Scheme 21).

![Chemical Structure](image)

Scheme 21. Toxicological implications for the equilibrium formation of SCEG from CEIC and GSH.
We elected to initiate our studies on the reactivity and toxicity of thiocarbamate conjugates using the GSH and NAC conjugates of CEIC for two reasons. Firstly, these compounds were identified as metabolites of BCNU, and secondly, some of the toxicities associated with BCNU therapy have been linked to its carbamoylating activity (Smith, 1989; Krell et al., 1991). The conjugates were examined with respect to their stability, and reactivity with model thiols. In buffered solution, SCEG exhibited a half-life of 5.0 h. This value is in the vicinity of $t_{1/2} = 5.9$ h, calculated from initial rate data reported for SCEG (Davis et al., 1993), and is close to the documented $t_{1/2}$ of 4.4 h for SMG (Pearson et al., 1990a). In the presence of NAC, SCEG concentration declined more rapidly ($t_{1/2}$ 44 min) reacting with the free thiol to form NCEC. In the converse situation, NCEC appeared less reactive in the presence of GSH, exhibiting a half-life of 3.7 h. In an earlier report of the reactivity of thiocarbamates in the presence of free thiols, cysteine conjugates were observed to be more reactive than their GSH counterparts (Pearson et al., 1990a). To account for this order of reactivity, it was proposed that the carboxylate anion of the cysteine conjugate functions as a base, and via an intramolecular abstraction of a proton from the carbamate nitrogen facilitates the E1cb process. The higher reactivity of SCEG over NCEC, in which the latter compound also has a free carboxylate, demonstrates that mechanism(s) other than the intramolecular participation of cysteine carboxylate as a base could contribute to the higher reactivity of cysteine over GSH thiocarbamate conjugates.

Compared to the half-life of SCEG in buffered solution, the reported value for BCNU ($t_{1/2}$, 49 min) under similar conditions (Weinkam et al., 1980) is 6-fold shorter. One possible explanation for the difference in the reactivity of the nitrosourea and thiocarbamate could reside in the chemistry of their functional groups (Figure 53). In BCNU, rearrangement through a 6-membered transition state proceeds with N-H cleavage and collapse of the nitrosourea ensemble into the isocyanate and diazonium hydroxide elements (Reed et al., 1975). Because the pKa lowering effect of the electron-withdrawing N-nitroso function (BCNU) is opposed by the electron-donating resonance of the glutathionyl S-substituent
(SCEG) (March, 1985b), the thiol conjugate is the less reactive of the two species. Considered altogether, these results demonstrate that the formation of thiocarbamate conjugates serves to extend the longevity of the carbamoylating activity of the parent nitrosourea.

![Chemical Structures](image)

Figure 53. Comparison of the structures of SCEG and BCNU to account for the superior stability of SCEG.

In order to investigate the carbamoylating activity of thiocarbamates in vivo, NCEC was administered to rats to characterize the formation of SCEG as a biliary metabolite. The rationale for the experimental design rested on the fact that the formation of SCEG could only conceivably arise through an equilibrium exchange of CEIC from NCEC to endogenous GSH, since metabolism of the NAC conjugate to its GSH analogue is unlikely. The identification of SCEG as a biliary metabolite substantiated the fact that the exchange of the isocyanate entity could take place in vivo (Figure 48); however, this result should be viewed with caution. Although the bile samples from this experiment were frozen after short durations of collection to minimize reaction of the thiocarbamate, collection over an acid buffered medium would have been more efficient in this respect (Experimental Section 5.1.2.2). Given the excretion of GSH (Madhu et al., 1993) and the parent compound NCEC into bile combined with the ambient alkalinity of the latter, it is possible that SCEG could have been formed "ex vivo" during the course of fluid collection. Collection of bile at acidic pH will verify whether the carbamoylating activity of the conjugate was indeed mediated in vivo.
5.2. Toxicity of thiocarbamate conjugates

It is conceivable that some of the pharmacological activity associated with nitrosoureas and formamides could be mediated by carbamoylated metabolites. The existence of BCNU (t$_{1/2}$ 14 min) and CCNU (t$_{1/2}$ 30 min) in serum is short (Levin et al., 1978; Weinkam et al., 1980), and can forseeably be further reduced in vivo with denitrosative metabolism by GSTs (Smith et al., 1989; Tuvesson et al., 1993; Weber and Waxman, 1993), P450 (Weber and Waxman, 1993), and NADPH-P450 reductase (Potter and Reed, 1982; 1983). The isocyanates arising from nitrosoureas (vide supra) and the P450 bioactivation of formamides (Hyland et al., 1992) are themselves fleetingly existent in aqueous systems (MIC, t$_{1/2}$ 2 min) (Brown et al., 1987). Thus, the carbamoylating activity of nitrosoureas and formamides can be seen as perpetuated in the form of thiocarbamate metabolites.

Several lines of evidence support the carbamoylating activity of thiocarbamate conjugates towards nucleophilic residues on biomolecules. With respect to structural biomolecules, SMG imparted the elements of MIC to nucleophilic groups on oxytocin and BSA (Pearson et al., 1991), and SCEG elicited the transfer of CEIC to the $\alpha$ and $\beta$-chains of human hemoglobin (Jochheim et al., 1994).

Studies presented here and by others (Kassahun et al., 1992; Davis et al., 1993; Guest and Varma, 1994) speak in favour of the thiocarbamate-mediated inactivation of functional proteins. In light of the inactivation of GR by BCNU and CEIC (Babson and Reed, 1978), and by the conjugate SMG (Jochheim and Baillie, 1994), our finding that SCEG is inhibitory toward mitochondrial GR suggests that this compound donates the isocyanate entity to the catalytic site of the enzyme either as the free electrophile released upon decomposition, or through the conjugate itself by a mechanism-based targeted delivery. Should prior decomposition of SCEG have been necessary to elicit this response, then only a small fraction of the conjugate would have been required, given the precipitous decline of GR activity and the slow rate of decomposition of SCEG (t$_{1/2}$ 5.0 h). Notwithstanding, the possibility of
SCEG decomposition catalyzed by mitochondrial protein cannot be excluded. Should intact SCEG have been the active agent, then it too could have diffused into the mitochondria, because the mitochondria, although nearly so (≥96%), were not completely intact. Transport mechanism(s) could also be operative for SCEG, as described for GSH itself (Martensson et al., 1990), although it should be noted in mitigation, that a transport system for GSSG has been excluded (Olafsdottir and Reed, 1988). Although evidence weighs in favour of carbamoylation as the mechanism for GR inactivation by nitrosoureas and thiocarbamates (vide supra), the involvement of active site alkylation has also been proposed for nitrosoureas (Karplus et al., 1988; Schallreuter et al., 1990). Apart from its carbamoylating activity, SCEG is also capable of alkylating guanosine (Stahl et al., 1992), and perhaps this activity could be contributory to its inhibition of GR.

The use of citrate synthase as an indicator of mitochondrial integrity when released by sonication underlines a noteworthy issue. This enzyme is not as vulnerable to SCEG inactivation (90% activity remaining at 6.25 h) as GR, and as such is suitable in this experiment as an intramitochondrial marker.

An important consequence of the inhibition of mitochondrial GR is the foreseeable elevation in mitochondrial GSSG due to H₂O₂ generated by aerobic respiration and the activity of glutathione peroxidase (see Introduction/Section 5.2). Since GSSG is not transported from mitochondria (Olafsdottir and Reed, 1988), this oxidant can be become disruptive to the mitochondrial milieu as suggested by Reed (1990). It was also proposed in the latter report that because of the absence of catalase in mitochondria, these organelles were dependent on GR for the regeneration of GSH from GSSG. Although this claim could not be verified for rat liver mitochondria in the recent literature, it has been demonstrated that rat heart mitochondria do contain catalase that is involved in alleviating the oxidative load of H₂O₂ (Radi et al., 1991; 1993). Should catalase indeed be present in rat hepatic mitochondria, the pivotal role cast for GR in the GSH redox cycle (Reed, 1990) could reside
in the superior efficiency of this system over catalase, given the lower $K_m$ of glutathione peroxidase for $H_2O_2$ (Chance et al., 1979).

As a part of our investigation into the toxicity of thiocarbamate conjugates, attention was focussed on their inhibitory properties toward oxidative phosphorylation. In the course of 90 min, SMG inhibited state III oxidative phosphorylation. As discussed above, it is not known whether mitochondrial pertubations elicited by thiocarbamates arise as a result of preliminary decomposition of the conjugate to release the isocyanate or whether transport mechanisms exist for the intact species. However, the block in respiration is viewed as attributable to the carbamoylating activity of SMG. This proposal is based on the observed concentration dependent inhibition of state III oxidative phosphorylation by MIC, ascribed to the impaired function of the complex I region of the electron transport chain (Jeevaratnam et al., 1992). Interestingly, mitochondrial respiration does not appear to be influenced by the status of mitochondrial GSH, since these organelles remained well coupled even when depleted of this thiol (Jocelyn and Cronshaw, 1985).

Envisioned as a corollary to reversible thiocarbamate formation is the hepatotoxicity of nitrosoureas (Colvin and Chabner, 1990) and formamides (Tulip and Timbrell, 1988; Redlich et al., 1990). The formation of GSH conjugates as biliary metabolites of CCNU and BCNU directs carbamoylating activity to the liver. BCNU has been shown to induce cholestasis due to a disruption of biliary tract integrity (Krell et al., 1991). Similar lesions have also been noted for CCNU in the short term, progressing to cholangiolyis, biliary cirrhosis and microtubule disruption of liver cells (Ducastelle et al., 1988). Biliary toxicity has also been reported for $N$-(1-methyl-3,3-diphenylpropyl)formamide (Mutlib et al., 1990). The carbamoylating challenge issued by GSH conjugates could very well be exacerbated by the relatively low levels of both GSH and GR in the biliary epithelial cell population (Parola et al., 1990). Indeed, it has been conjectured that the biliary pathogenesis of 1-naphthylisothiocyanate, a structural analogue to MPIC, is linked to its GSH conjugate which acts as a latent form of the isothiocyanate (Carpenter-Deyo et al., 1991). In this respect, it is
noteworthy that the nitrosourea fotemustine stands apart from both BCNU and CCNU, being devoid of such hepatotoxic manifestations (Laquerriere et al., 1991). It is possible that this property of fotemustine could reside in its weak carbamoylating activity toward tissue GR in the rat and in isolated hepatocytes (Boutin et al., 1989). This could have some bearing on the metabolism of fotemustine (vide supra). Whereas, BCNU and CCNU are metabolized to carbamoyl conjugates, no such metabolites have been characterized for fotemustine (Brakenhoff et al., 1993).

Thus, the formation of thiocarbamate conjugates as metabolites of nitrosoureas and formamides does not eliminate the activity of isocyanates, but instead, paradoxically extends their lifetime. In view of the widespread activity of these conjugates to biological systems, this area of research is crucial to the understanding of the pharmacology of their parent compounds.
SUMMARY AND CONCLUSIONS

Although chemically dissimilar, nitrosoureas and formamides share the common feature of acting as precursors of reactive, electrophilic isocyanate species. Whereas nitrosoureas spontaneously decompose to isocyanates, formamides are converted by metabolism. Irrespective of the mechanism, the formation of isocyanates was considered the underlying cause of some toxicities associated with these two groups of xenobiotics. Consequently, the research of this dissertation was designed to test this hypothesis.

Carbamoylated GSH and NAC conjugates were synthesized as reference compounds for potential metabolites of CCNU, BCNU and NFA, and their structural properties studied by LC/MS/MS. Information on these fragmentations was exploited in the identification of metabolites both in vivo and in vitro by either full scan LC/MS/MS or SRM experiments.

CCNU was converted in rats to 4-hydroxycyclohexyl, 3-hydroxycyclohexyl and cyclohexyl isocyanate which were excreted as GSH and NAC conjugates in bile and urine, respectively. The NAC conjugates corresponding to these isocyanates, namely NFHC, NTHC and NCCC constituted 1.6 ± 0.3, 9.2 ± 1.4 and 3.5 ± 1.3 % (14.3 ± 2.9 % total carbamoyl NAC conjugates) of the CCNU dose excreted in 24 h. The GSH-dependent metabolism of CCNU was also evident in two patients on chemotherapy with the detection of NFHC, NTHC and NCCC as urinary metabolites. These results indicate that despite the rapid P450 hydroxylation to which CCNU is reported to be subject in vivo (Hilton and Walker, 1975a; 1975b), the parent drug also contributes to carbamoylating activity alongside its hydroxylated derivatives.

GSH and NAC conjugates of CEIC were also characterized as metabolites of both BCNU and CEIC in the bile and urine, respectively, of dosed rats. This lent support to the contention that the isocyanate formed from the decomposition of the nitrosourea contributes to the carbamoylating activity of the parent drug. The urinary NAC conjugate
of CEIC was quantitated in five patients on BCNU chemotherapy and concentrations varied from 5.0 to 13.6 nmol/mL.

LC/MS and LC/MS/MS in conjunction with stable isotope methodology provided a valuable approach to the detection and ultimate characterization of NFA-derived thiol conjugates. Apart from the expected identification of carbamoylated GSH and NAC conjugates as biliary and urinary metabolites, respectively, cysteinylglycine, cysteine and NAC conjugates were characterized as novel formamide metabolites in bile. Mechanisms by which this excretory profile could arise were proposed.

The metabolism of NFA by rat hepatic subcellular fractions was investigated to characterize the organelles which catalyze bioactivation to MPIC. The product was trapped as the GSH conjugate SMPG and a LC/MS method was developed for the detection of the intact conjugate by SRM. It appears that P450 is involved in the metabolism of NFA to SMPG by microsomes, based on the 63 % inhibition of this reaction by metyrapone and the dependence of catalysis on NADPH. Microsomal metabolism of NFA to SMPG appeared to be modestly induced by PB but not by acetone. This finding, coupled with the fact that neither DEDTC nor orphenadrine appreciably inhibits microsomal SMPG formation, speaks for a minor role for catalysis by P450 2E1 and 2B1/2B2. The catalytic isoform(s) involved in this process await characterization.

Metabolism of NFA by intact mitochondria revealed the formation of SMPG as a product. Thus, efforts were directed toward establishing a role for mitochondrial P450 by investigating the catalytic process when supported by NADPH in sonicated mitoplasts. However, significant levels of microsomal contamination were detected in mitoplast preparations, and so the conversion of NFA to SMPG in NADPH-supported mitoplasts could not be solely attributed to mitochondrial P450. Consequently, the precise mechanism by which NFA is bioactivated to MPIC in intact mitochondria remains unclear.
The GSH and NAC conjugates of CEIC were examined with respect to their reactivity and toxicity toward mitochondrial enzymatic activities. The conjugates decomposed under physiological conditions and were found to donate the isocyanate entity to thiol nucleophiles in vitro. The discovery of SCEG as a biliary metabolite of NCEC in rats was considered to be reflective of a similar exchange process of CEIC between the administered NAC conjugate and endogenous GSH. SCEG was found to be potently inhibitory to mitochondrial GR, and SMG blocked oxidative phosphorylation. These lines of evidence together suggest that thiocarbamate conjugates are themselves toxic and act as latent sources of carbamoylating activity.

In conclusion, it is appropriate to evaluate the contribution of this research from the perspective of its overall goal to investigate the involvement of isocyanates in the metabolism of nitrosoureas (CCNU and BCNU) and high molecular weight formamides (NFA). Compelling evidence for the involvement of these xenobiotics as precursors of isocyanates in vivo was obtained in the form of GSH-dependent metabolites, suggesting that the depletion of this important thiol could be a mechanism of toxicity. Furthermore, it was demonstrated that thiocarbamate conjugates are themselves reactive species and disrupt the activity of mitochondrial enzymes. These results support the assertion that the conjugation of isocyanates to thiols is not necessarily a detoxification pathway, but paradoxically extends the longevity of the carbamoylating activity of the isocyanate. Indeed, it is quite likely that these thiol metabolites to some extent mediate the delayed systemic toxicities associated with nitrosoureas and formamides.
VI. REFERENCES


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VII. APPENDIX

Listing of compounds frequently referred to in the text.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
<th>Structure</th>
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<tbody>
<tr>
<td>BCNU</td>
<td>1,3-bis(2-chloroethyl)-1-nitrosourea</td>
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<tr>
<td>CCNU</td>
<td>1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea</td>
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<tr>
<td>CEIC</td>
<td>2-chloroethyl isocyanate</td>
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<tr>
<td>MIC</td>
<td>methyl isocyanate</td>
<td>CH₃-N=C=O</td>
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<tr>
<td>MPIC</td>
<td>1-methyl-2-phenylethyl isocyanate</td>
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<td>NCCC</td>
<td>N-acetyl-S-(cyclohexylcarbamoyl)-cysteine</td>
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<tr>
<td>NCEC</td>
<td>N-acetyl-S-[(2-chloroethyl)carbamoyl]-cysteine</td>
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<tr>
<td>NFA</td>
<td>N-formyl amphetamine</td>
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<tr>
<td>NFHC</td>
<td>N-acetyl-S-[(4-hydroxycyclohexyl)carbamoyl]cysteine</td>
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<td>NMF</td>
<td>N-methylformamide</td>
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<td>NMPC</td>
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<td>NMPG</td>
<td>N-[(1-methyl-2-phenylethyl)carbamoyl]-glutathione</td>
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Listing of compounds frequently referred to in the text (cont'd).

<table>
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<th>Abbreviation</th>
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<th>Structure</th>
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<td>NTHC</td>
<td>(N)-acetyl-(S)-[(3-hydroxycyclohexyl)-carbamoyl]cysteine</td>
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<td>SCCG</td>
<td>(S)-[(cyclohexylcarbamoyl)glutathione</td>
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</tr>
<tr>
<td>SCEG</td>
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<td></td>
</tr>
<tr>
<td>SFHG</td>
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<td></td>
</tr>
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<td>SMG</td>
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<tr>
<td>SMPC</td>
<td>(S)-[(1-methyl-2-phenylethyl)carbamoyl]-cysteine</td>
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</tr>
<tr>
<td>SMPCG</td>
<td>(S)-[(1-methyl-2-phenylethyl)carbamoyl]-cysteinylglycine</td>
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<tr>
<td>SMPG</td>
<td>(S)-[(1-methyl-2-phenylethyl)carbamoyl]glutathione</td>
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</tr>
<tr>
<td>STHG</td>
<td>(S)-[(3-hydroxycyclohexyl)carbamoyl]glutathione</td>
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