THERAPEUTIC MECHANISMS OF PROPYLTHIOURACIL IN THE TREATMENT OF ALCOHOLIC LIVER DISEASE

by

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A Thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmacology
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ABSTRACT

Alcoholic liver disease (ALD) is a common and serious disorder that responds poorly to treatment with conventional pharmacological therapies. The antithyroid drug propylthiouracil (PTU) has been shown to ameliorate ALD in both rodents and humans, however the mechanism of action has not been clearly defined. The present studies examined several possible mechanisms that may contribute to the cytoprotective properties of PTU.

Induction of the microsomal enzymes cytochrome P4502E1 (CYP2E1) and NADPH-cytochrome P450 reductase (CYP-reductase) may be involved in the pathogenesis of ALD, since they can generate free radicals and reactive metabolites, and contribute to oxygen depletion. Initial studies examined the potential of PTU to inhibit CYP-reductase, a rate-limiting enzyme in P450-catalyzed reactions, as a potential mechanism underlying the hepatoprotective effect of PTU. Liver microsomes isolated from chronic ethanol-treated rats showed increased levels of CYP2E1 and enhanced rates of oxygen consumption, hydroxyl radical formation and ethanol oxidation. These effects were attenuated in the microsomes of rats treated with PTU, in parallel with reductions in the levels and activity of CYP-reductase.

Neutrophil infiltrates in the liver and elevated levels of circulating neutrophils are characteristic of ALD. Neutrophils can injure host cells by releasing reactive oxygen species and proteolytic enzymes. Studies were conducted to determine whether PTU could lower circulating neutrophil counts and thereby protect against liver injury. Chronic intragastric ethanol infusion resulted in liver injury accompanied by an increase in circulating neutrophils. Administration of PTU to ethanol-fed rats did not alter circulating neutrophil counts.

Studies further examined the potential of PTU to modulate neutrophil oxidant formation and proteolytic enzyme activity. Myeloperoxidase is a neutrophil enzyme that generates reactive oxygen species and renders cells more susceptible to proteolytic injury by inactivating α1-
proteinase inhibitor (A1PI). Studies with rat neutrophils showed that, at clinically relevant concentrations, PTU was a potent inhibitor of myeloperoxidase and prevented both the formation of hypochlorous acid and the inactivation of A1PI by neutrophils.

These studies indicate that the ability of PTU to (a) counteract the induction of CYP2E1 via CYP-reductase down-regulation and (b) limit the toxic potential of neutrophils are probable mechanisms contributing to the drug's therapeutic effect in ALD patients.
This thesis is dedicated in loving memory to Morton Ross (1933–1995),
my father, friend and teacher.
ACKNOWLEDGMENTS

I wish to express my greatest thanks to Dr. Yedy Israel for sharing his wisdom and for his encouragement and friendship over the years.

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Special thanks for the many good times shared with my brothers from the old neighborhood - Mr. James Stuart Lyon, Mr. Glen Avigdor and Mr. Fraser Paterson, my friends at the University of Toronto - Dr. Mayank Patel, Dr. Adriano Marchese, Dr. C. Moncada and Mrs. Veronica Torres, and my band mates from Thomas Jefferson University and the very soulful Perfect Body - Dr. Richard Horn, Mr. Walter Peeler, Mr. Dayan Hall, Mr. Alfonso Curry, Miss Rhonda Lancaster and Mr. Gary Rice.

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xiii</td>
</tr>
<tr>
<td>1. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Characteristics of Alcoholic Liver Disease</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1 Morphological Features</td>
<td>2-4</td>
</tr>
<tr>
<td>1.1.1.1 Fatty Liver (Steatosis)</td>
<td>2</td>
</tr>
<tr>
<td>1.1.1.2 Alcoholic Hepatitis</td>
<td>3</td>
</tr>
<tr>
<td>1.1.1.3 Alcoholic Cirrhosis</td>
<td>4</td>
</tr>
<tr>
<td>1.1.1.4 Other Morphological Features</td>
<td>4</td>
</tr>
<tr>
<td>1.1.2 Clinical and Biochemical Characteristics</td>
<td>5-6</td>
</tr>
<tr>
<td>1.1.3 Incidence and Prognosis</td>
<td>6</td>
</tr>
<tr>
<td>1.2 Mechanisms of Ethanol Hepatotoxicity</td>
<td>7</td>
</tr>
<tr>
<td>1.2.1 Free Radicals and Reactive Oxygen Species</td>
<td>8-38</td>
</tr>
<tr>
<td>1.2.1.1 Free Radical Biochemistry</td>
<td>8</td>
</tr>
<tr>
<td>1.2.1.2 Biological Sources and Targets of Free Radicals</td>
<td>11</td>
</tr>
<tr>
<td>1.2.1.3 Oxidative Stress and Antioxidant Defences</td>
<td>13</td>
</tr>
<tr>
<td>1.2.1.4 Ethanol and Oxidative Stress</td>
<td>15</td>
</tr>
<tr>
<td>1.2.2 Oxygen Depletion and Liver Hypoxia</td>
<td>21</td>
</tr>
<tr>
<td>1.2.3 Acetaldehyde</td>
<td>23</td>
</tr>
<tr>
<td>1.2.4 Alcohol Dehydrogenase</td>
<td>24</td>
</tr>
<tr>
<td>1.2.5 Aldehyde Dehydrogenase</td>
<td>26</td>
</tr>
<tr>
<td>1.2.6 Cytochrome P4502E1</td>
<td>28</td>
</tr>
<tr>
<td>1.2.6.1 Role of Cytochrome P4502E1 in Ethanol Hepatotoxicity</td>
<td>33</td>
</tr>
<tr>
<td>1.2.7 NADPH-Cytochrome P450 Reductase</td>
<td>36</td>
</tr>
<tr>
<td>1.2.8 Neutrophils</td>
<td>38</td>
</tr>
<tr>
<td>1.2.8.1 Neutrophil-Derived Oxidants</td>
<td>40</td>
</tr>
<tr>
<td>1.2.8.2 Proteolytic Enzymes and Serum α-1-Proteinase Inhibitor</td>
<td>44</td>
</tr>
<tr>
<td>1.2.8.3 Role of Neutrophils in Alcoholic Liver Disease</td>
<td>45</td>
</tr>
</tbody>
</table>
# 1.3 Therapy of Alcoholic Liver Disease

1.3.1 General Pharmacology of Propylthiouracil
1.3.2 Hepatoprotective Effect of Propylthiouracil
1.3.3 Putative Mechanisms of Propylthiouracil Hepatoprotection
1.3.4 Effect of Propylthiouracil on NADPH-Cytochrome P450 Reductase
1.3.5 Effects of Propylthiouracil on Neutrophils
   1.3.5.1 Effect of Propylthiouracil on Neutrophil Count
   1.3.5.2 Effect of Propylthiouracil on Neutrophil Myeloperoxidase

# 2. AIMS OF THE INVESTIGATION

# 3. STUDY 1: EFFECT OF PROPYLTTHIOURACIL TREATMENT ON NADPH-CYTOCHROME P450 REDUCTASE LEVELS, OXYGEN CONSUMPTION AND HYDROXYL RADICAL FORMATION IN LIVER MICROSONES FROM RATS FED ETHANOL OR ACETONE CHRONICALLY.

## 3.1 Rationale

## 3.2 Method

3.2.1 Materials
3.2.2 Chronic Ethanol Administration
3.2.3 Chronic Acetone Administration
3.2.4 Surgical Procedures and Isolation of Liver Microsomes
3.2.5 Analytical Procedures
   3.2.5.1 Thyroid Hormones
   3.2.5.2 Protein Determination
   3.2.5.3 Microsomal Oxygen Consumption
   3.2.5.4 Total P450
   3.2.5.5 CYP-reductase Activity
   3.2.5.6 NDMA Oxidation
   3.2.5.7 MEOS Activity
   3.2.5.8 Microsomal OH\(^{-}\) Formation
   3.2.5.9 Western Blot Determination of CYP2E1 and CYP-reductase
3.2.6 Statistical Analysis

## 3.3 Results

3.3.1 Chronic Ethanol/PTU Study
3.3.2 Chronic Acetone/PTU Study

## 3.4 Discussion
4. STUDY 2: EFFECT OF PROPYLTHIOURACIL AND GRANULOCYTE COLONY STIMULATING FACTOR ON CIRCULATING NEUTROPHIL COUNTS AND LIVER INJURY IN RATS RECEIVING ETHANOL ORALLY OR BY INTRAGASTRIC INFUSION.

4.1 Introduction

4.2 Method
   4.2.1 Oral Ethanol Feeding Model
   4.2.2 Continuous Intragastric Ethanol Infusion Model
   4.2.3 Propylthiouracil Treatment
   4.2.4 G-CSF Treatment
   4.2.5 Blood Biochemistry and Cell Count
   4.2.6 Liver Histology
   4.2.7 Statistics

4.3 Results
   4.3.1 Oral Ethanol Feeding
   4.3.2 Intragastric Ethanol Infusion
   4.3.3 Propylthiouracil Treatment
   4.3.4 G-CSF Treatment

4.4 Discussion
   4.4.1 Neutrophils in Rat Models of Alcoholic Liver Disease
   4.4.2 Neutropenia as a Mechanism of Propylthiouracil Hepatoprotection?

5. STUDY 3: EFFECT OF ANTITHYROID DRUGS ON HYDROXYL RADICAL FORMATION AND \( \alpha \)-1-PROTEINASE INHIBITOR INACTIVATION BY NEUTROPHILS: THERAPEUTIC IMPLICATIONS.

5.1 Introduction

5.2 Method
   5.2.1 Reagents
   5.2.2 Rat Neutrophil Isolation
   5.2.3 Chicken Neutrophil Isolation
   5.2.4 Hypochlorous Acid Determination
   5.2.5 Superoxide Determination
   5.2.6 AIPI Activity
   5.2.7 Electron Spin Resonance/Spin Trapping of \( \alpha \)-hydroxyethyl Radicals
   5.2.8 Cytotoxicity
   5.2.9 Statistics

5.3 Results
5.4 Discussion

5.4.1 A Role for Myeloperoxidase and Hypochlorous Acid in Hydroxyl Radical Generation?

6. GENERAL DISCUSSION

7. DIRECTIONS FOR FURTHER RESEARCH

7.1 Clinical Trials of Propylthiouracil for the Treatment of Alcoholic Liver Disease

7.2 Propylthiouracil Therapy in the Treatment of Other Forms of Tissue Injury

7.3 Propylthiouracil as a Prototype for the Development of Novel Therapeutic Agents

7.4 Role of Peroxynitrite in the Generation of Hydroxyl Radicals

8. REFERENCES

9. APPENDICES

9.1 Appendix 1. Recipes for Liquid Diets in Ethanol Administration Studies

9.2 Appendix 2. Effect of Propylthiouracil on Thyroid Hormones and Cytochrome P450 reductase in Rats: A Dose-Response Study

9.3 Appendix 3. Effect of Propylthiouracil on Hepatic Non-heme Iron Content in Chronic Ethanol-Treated Rats

9.4 Appendix 4. Antisense Inhibition of Myeloperoxidase: A Novel Strategy for the Treatment of Inflammatory Tissue Injury
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemical Structures of the Antithyroid Drugs Thiourea, Propylthiouracil and Methimazole.</td>
<td>51</td>
</tr>
<tr>
<td>2.</td>
<td>Effect of PTU on NADPH-cytochrome P450 reductase activity in liver microsomes from ethanol-treated rats.</td>
<td>75</td>
</tr>
<tr>
<td>3.</td>
<td>Immunoblot analysis of NADPH-cytochrome P450 reductase content in liver microsomes isolated from rats receiving PTU and chronic ethanol.</td>
<td>76</td>
</tr>
<tr>
<td>4.</td>
<td>Effect of chronic ethanol administration and PTU treatments on the rate of NADPH-dependent oxygen consumption in rat liver microsomes.</td>
<td>78</td>
</tr>
<tr>
<td>5.</td>
<td>Effect of chronic ethanol and PTU treatments on the rate of microsomal production of hydroxyl radicals.</td>
<td>79</td>
</tr>
<tr>
<td>6.</td>
<td>Immunoblot analysis of cytochrome P4502E1 content in liver microsomes isolated from rats receiving PTU and chronic ethanol.</td>
<td>82</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of PTU on NADPH-cytochrome P450 reductase activity in liver microsomes from acetone-treated rats.</td>
<td>85</td>
</tr>
<tr>
<td>8.</td>
<td>Effect of chronic acetone and PTU administration on the rate of NADPH-dependent oxygen consumption in rat liver microsomes.</td>
<td>86</td>
</tr>
<tr>
<td>9.</td>
<td>Effect of chronic acetone and PTU treatments on the rate of microsomal production of hydroxyl radicals.</td>
<td>87</td>
</tr>
<tr>
<td>10.</td>
<td>Effect of oral ethanol feeding on indices of liver injury in rats.</td>
<td>101</td>
</tr>
<tr>
<td>11.</td>
<td>Effect of chronic intragastric ethanol infusion on indices of liver injury in rats.</td>
<td>104</td>
</tr>
<tr>
<td>12.</td>
<td>Effect of propylthiouracil on circulating neutrophil counts in rats receiving continuous intragastric infusion of control liquid diets.</td>
<td>105</td>
</tr>
<tr>
<td>13.</td>
<td>Effect of propylthiouracil on circulating neutrophil counts in rats receiving continuous intragastric infusion of ethanol-containing liquid diets.</td>
<td>106</td>
</tr>
<tr>
<td>15.</td>
<td>Effect of inhibitors on hypochlorous acid formation by PMA-stimulated rat neutrophils.</td>
<td>120</td>
</tr>
<tr>
<td>16.</td>
<td>Effect of inhibitors on superoxide formation by PMA-stimulated rat neutrophils.</td>
<td>122</td>
</tr>
</tbody>
</table>
17. Effect of inhibitors on neutrophil-mediated AlPI inactivation. 123
18. ESR/4-POBN spin trapping of α-hydroxyethyl radicals in PMA-stimulated rat neutrophils. 124
19. ESR/4-POBN spin trapping of α-hydroxyethyl radicals in PMA-stimulated chicken neutrophils. 126
20. Pilot study on the effect of PTU on serum T₄ concentration in rats. 193
21. Pilot study on the effect of PTU on serum free thyroxine index in rats. 194
22. Pilot study on the effect of propylthiouracil (PTU) on hepatic microsomal cytochrome P450-reductase activity in rats. 195
23. Effect of propylthiouracil on hepatic non-heme iron content in ethanol-treated and control rats. 199
LIST OF TABLES

Table | Page
-----|------
1. Substrates and inducers of cytochrome P450E1. | 32
2. Effects of chronic ethanol and PTU treatments on body weight gain and serum levels of thyroid hormones. | 74
3. Effects of ethanol and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA. | 80
4. Effects of chronic acetone and PTU treatments on body weight gain, liver weight and serum thyroid hormone levels. | 83
5. Effects of acetone and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA. | 88
6. Hematological parameters in rats fed ethanol-containing or isocaloric control liquid diets orally for 30 days. | 100
7. Hematological parameters in rats receiving ethanol-containing or isocaloric control liquid diets by intragastric infusion for 87 days. | 102
8. Effect of daily treatment with G-CSF on hematological parameters in rats fed ethanol-containing or isocaloric control liquid diets orally for 30 days. | 108
9. Myeloperoxidase antisense target sequences and complementary antisense sequences. | 210
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1PI</td>
<td>α-1-proteinase inhibitor</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase (EC 1.1.1.1)</td>
</tr>
<tr>
<td>ALD</td>
<td>alcoholic liver disease</td>
</tr>
<tr>
<td>AH</td>
<td>alcoholic hepatitis</td>
</tr>
<tr>
<td>a_h</td>
<td>hydrogen hyperfine splitting constant</td>
</tr>
<tr>
<td>a_n</td>
<td>nitrogen hyperfine splitting constant</td>
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<td>aldehyde dehydrogenase (EC 1.2.1.3)</td>
</tr>
<tr>
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<td>alanine aminotransferase (EC 2.6.1.2)</td>
</tr>
<tr>
<td>ANOVA</td>
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</tr>
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<td>aspartate aminotransferase (EC 2.6.1.1)</td>
</tr>
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<td>ATP</td>
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</tr>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
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</tr>
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<td>Cu/ZnSOD</td>
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</tr>
<tr>
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</tr>
<tr>
<td>CYP2E1</td>
<td>cytochrome P4502E1 (EC 1.5.99)</td>
</tr>
<tr>
<td>DFO</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>d-PBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>DPI</td>
<td>diphenyleneiodonium chloride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
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<td>ESR</td>
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</tr>
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</tr>
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</tr>
<tr>
<td>G</td>
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</tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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<td>glutathione peroxidase</td>
</tr>
<tr>
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<td>hemoglobin</td>
</tr>
<tr>
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<td>Hank’s balanced salt solution</td>
</tr>
<tr>
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<td>hydroxyethyl radical</td>
</tr>
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<td>HOCl</td>
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</tr>
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<td>IC_{50}</td>
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</tr>
<tr>
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</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
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</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
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<td>kilodalton</td>
</tr>
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<td>MEOS</td>
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</tr>
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</tr>
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</tr>
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</tr>
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</tr>
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<td>MMI</td>
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</tr>
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<td>myeloperoxidase (EC 1.11.1.7)</td>
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</tr>
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<td>NADP$^+$</td>
<td>oxidized nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>NADPH-FMN oxidoreductase (EC 1.6.99.1)</td>
</tr>
<tr>
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<td>nanometer</td>
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<td>NO$^+$</td>
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<td>$O_2^-$</td>
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</tr>
<tr>
<td>$OH^+$</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
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</tr>
<tr>
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<td>prostaglandin E_2</td>
</tr>
<tr>
<td>4-POBN</td>
<td>4-pyridyl 1-oxide N-tert-butyl nitrone</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear leukocyte or neutrophil</td>
</tr>
<tr>
<td>p.o.</td>
<td>per os</td>
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<tr>
<td>PTU</td>
<td>propylthiouracil</td>
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<td>RBC</td>
<td>red blood cell</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<td>second</td>
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<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SLAPN</td>
<td>N-succinyl-L-(ala)_3-p-nitroanilide</td>
</tr>
<tr>
<td>T_3</td>
<td>triiodothyronine</td>
</tr>
<tr>
<td>T_4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TNB</td>
<td>5-thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>TPO</td>
<td>thyroid peroxidase (EC 1.11.1.7)</td>
</tr>
<tr>
<td>U</td>
<td>units</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
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<tr>
<td>μm</td>
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</tr>
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<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>XDH</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
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<tr>
<td>WBC</td>
<td>white blood cell</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
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1. INTRODUCTION

Alcoholic liver disease (ALD) is the most common and serious health complication directly associated with excessive consumption of alcohol. Chronic liver disease and cirrhosis, of which alcohol abuse is the principal cause, is the tenth leading cause of death in Canada accounting for 7.3% of overall mortality in 1994 (Statistics Canada, 1994). In Canada and the United States an estimated 1000 and 11,000 deaths, respectively, were directly attributable to ALD (Single et al., 1995; Caces et al., 1995). In Canada alone, the cost of treatment for ALD is substantial as these patients required over 78,000 days of hospitalization annually (Single et al., 1995).

Despite considerable advances in the past two decades in elucidating the pathogenic mechanisms of ALD, there has been little progress in the development of effective therapies for the disease and the prognosis after initial diagnosis remains poor. In general, one third to two-thirds of patients with alcoholic hepatitis and/or cirrhosis die within 5 years of diagnosis (Finlayson, 1993).

Propylthiouracil (PTU), a commonly used antithyroid drug, is one of the few agents shown to be effective in treating ALD (Orrego et al., 1987). Experimental studies in animals and clinical trials in humans have reported that PTU reduces both the severity and lethality of alcohol-induced liver injury. Despite these promising findings, a lack of information regarding the therapeutic mechanism of PTU has hampered its acceptance as a viable treatment option for the ALD patient.

The objective of the experiments presented herein was to elucidate the biochemical mechanisms underlying the hepatoprotective effect of PTU. The theoretical basis for these studies is presented in detail in the following general review of the characteristics and suspected
mechanisms of alcoholic liver disease and of the prior experimental evidence pertaining to the therapeutic properties of PTU.

1.1 Characteristics of Alcoholic Liver Disease (ALD)

1.1.1 Morphological Features

The morphological abnormalities characteristic of ALD develop in three stages which are classified on the basis of light microscopy. These are, in increasing order of severity: fatty liver, hepatitis and cirrhosis. The disease generally develops sequentially but there is often a considerable overlap in histological abnormalities among the stages. The definitive diagnostic features of ALD have been thoroughly characterized and described in several past reviews (Finlayson, 1993; French et al., 1993b; Harrison and Burt, 1993; International Group, 1987; Ishak et al., 1991; Christoffersen and Poulsen, 1979; Edmonson, 1986).

1.1.1.1 Fatty Liver: Fat accumulation (steatosis) in the liver represents the first stage of ALD and is the mildest and most common manifestation of ethanol hepatotoxicity in humans. In alcoholic steatosis the fat content of the liver can increase dramatically from 5% to 50% over the normal content of 5% fat by weight (Harrison and Burt, 1993). Fat deposition is observed in the form of small (microvesicular) intracellular fat droplets of 1-3 μm in diameter, a condition referred to as foamy degeneration. These small fat droplets often coalesce to form large (macrovesicular) droplets, with displacement of the cytoplasm and nucleus as a result. Typically, a mixture of both types of steatosis is present in the liver of alcoholics. Fat droplets from adjacent cells may coalesce to form larger fat cysts. Lipogranulomas, formed when fat cysts rupture and become surrounded by inflammatory infiltrates, are often observed in patients with alcoholic steatosis. Alcoholic steatosis can occur following acute ethanol ingestion and is common in heavy
drinkers. While steatosis is the earliest histological evidence of ALD, it is considered a reversible lesion and is not necessarily a precursor to more severe forms of ALD (Thaler, 1975).

1.1.1.2 Alcoholic Hepatitis: Alcoholic hepatitis (AH - or steatonecrosis), the second stage of ALD, is a much more serious condition than alcoholic fatty liver and is associated with more pronounced histopathological changes that mainly affect zone 3 of the hepatic acinus. Microscopically, AH is characterized by ballooning degeneration or swelling of hepatocytes and hepatocyte necrosis. Death of the hepatocyte is associated with nuclear and cell membrane lysis and elicits an inflammatory response which includes neutrophils, monocytes and lymphocytes. The migration of neutrophils into necrotic cells and their degranulation is thought to be one of the factors responsible for hepatocellular damage in ALD. Parenchymal fibrosis, in the form of fine collagen fibres surrounding centrilobular hepatocytes, is another hallmark characteristic of AH. Alcoholic fibrosis is characterized by accumulation of type I and type III collagen, laminin, fibronectin and elastase fibres. Deposition occurs initially in the Space of Disse and is accompanied by the development of a basement membrane under the fenestrated sinusoidal epithelium, a process known as capillarization (Schaffner and Popper, 1963). Later the connective tissue condenses and becomes a confluent mass that produces obliteration of sinusoids and central veins and occlusive lesions of the terminal hepatic venules (sclerosing hyaline necrosis). Mallory bodies (alcoholic hyaline) formed from the cross-linking of cytokeratin proteins in the cytoskeleton of liver cells (Ishak et al., 1991) are also commonly observed in AH.

Although AH is generally regarded as an intermediate stage leading to cirrhosis, fibrosis and cirrhosis may occur in the absence of widespread necrosis and inflammation (Nakano et al., 1982).
1.1.1.3 **Alcoholic Cirrhosis**: Alcoholic cirrhosis is the end-stage of ALD and is characterized by extensive fibrosis and the conversion of normal liver architecture into abnormal nodules (Anthony *et al.*, 1983), accompanied by functional decline. Macroscopically the early cirrhotic liver is golden yellow with fine uniform nodules of 1-5 mm in diameter. In later stages the size of the nodules increases to 5-50 mm and deep scars appear. The size of the liver varies depending on the degree of coexisting fibrosis, inflammation and steatosis, from a small shrunken and hard liver to a large organ weighing up to 4 kg. Microscopically, cirrhosis is characterized by abnormal remodeling of the extracellular matrix. Scar tissue distorts the normal architecture of the liver by forming bands of connective tissue joining portal and central zones, eventually leading to complete encirclement of islets of hepatocytes (Hall, 1987). Hepatic blood flow is often impaired in the cirrhotic liver due to the formation of regenerative nodules which compress the hepatic sinusoids. Histologically, there is often complete obliteration of terminal hepatic veins by fibrous tissue (Harrison and Burt, 1993).

1.2.1.4 **Other Morphological Features**: The proliferation of bile ducts and the presence of bile pigments associated with cholestasis, are evident in some patients, particularly those with coexisting alcoholic pancreatitis. Hepatocellular carcinoma may supervene in 5-15% of patients with alcoholic cirrhosis. Iron deposits (siderosis) both in parenchymal and Kupffer cells and increased tissue levels of copper (Berresford *et al.*, 1980), are found in the livers of many patients with ALD, particularly when cirrhosis is present. Examination by electron microscopy often reveals enlargement of mitochondria (megamitochondria) and smooth endoplasmic reticulum, an increase in the total non-organelle cytoplasmic volume, and a decrease in glycogen (Chen *et al.*, 1987).
1.1.2 Clinical and Biochemical Characteristics

The clinical features of ALD are indicative of injury to the hepatic parenchyma and resulting hepatic insufficiency, distortion of the portal circulation or a combination of both. In addition, extrahepatic factors associated with alcoholism, such as gastrointestinal, renal, endocrine, circulatory, hematological and neurological complications, may also contribute to the clinical spectrum of ALD.

Most individuals with alcoholic steatosis are asymptomatic, however hepatomegaly and jaundice may be evident in some cases. Clinical features typical of AH and cirrhosis include malaise, anorexia, weight loss, upper abdominal pain, hepatic enlargement and tenderness, diarrhea, fever, jaundice, spider telangiectasia, palmar erythema, gynaecomastia and neutrophil leukocytosis. In patients with alcoholic hepatitis and/or cirrhosis, occlusive injury to hepatic vessels, and resulting portal hypertension, may result in esophageal varices and splenomegaly/hypersplenism due to abdominal collateral circulation. Ascites and encephalopathy develop in many cases and there may be a progression to fulminant hepatic failure. Susceptibility to infection is increased in many ALD patients, particularly in the later stages of the disease.

Serum levels of the enzymes aspartate aminotransferase (AST: EC 2.6.1.1) and alanine aminotransferase (ALT: EC 2.6.1.2) are currently the most reliable and widely accepted biochemical indices used in the diagnosis and management of ALD. The levels of AST and ALT may be slightly elevated (< 200%) in the serum of some patients with alcoholic steatosis. The levels of AST are elevated in more than 90% of patients with AH, although values rarely exceed eight times the normal. Serum AST levels usually return to normal within 1-4 weeks of abstinence depending on the severity of ALD (Skude and Wadstein, 1977). Elevations in ALT also occur, but to a lesser extent, and therefore the ratio of AST:ALT may typically range from 8:1 to 2:1. A suggested criteria of AST/ALT > 1 and AST + ALT < 300 U/l correctly identified ALD in 75%
of patients (sensitivity) and incorrectly identified non-alcoholic liver disease in 33% (specificity) (Ryback et al., 1982).

Other common biochemical abnormalities in ALD include hypoalbuminemia and hyperglobulinemia (which are seen in about 70% and 50% of AH patients respectively), lipidemia and elevated levels of creatinine and alkaline phosphatase. Plasma electrolyte imbalances are also common and include decreases in potassium, sodium and to a lesser extent calcium and magnesium (Chiba et al., 1987). In more severe cases of ALD, clotting abnormalities and elevated serum bilirubin may also be observed.

1.1.3 Incidence and Prognosis

The risk of developing ALD increases in direct proportion to the amount of ethanol consumed and the duration of consumption. Intake in excess of 80 g (approximately 6 typical drinks\(^1\)) of alcohol per day is associated with a 52% incidence of cirrhosis in men over a 20 year period. This level has been defined as “hazardous drinking” (Rankin et al., 1975) since consumption of amounts in excess of that are associated with progressive increases in prevalence and severity of liver cirrhosis (Lelbach, 1975). More recent studies have suggested risk levels as low as 40 g of ethanol per day for men (Batey et al., 1992) and 20 g per day for women (Tuyns and Pequignot, 1984).

While reports vary considerably, it has been estimated that 80% of heavy consumers of alcohol develop fatty liver, only 10-35% develop clinical or histological signs of hepatitis and 8-30% develop cirrhosis (Grant et al., 1988). AH appears in patients who have been consuming excess amounts of ethanol for periods of 5 years or more (rarely as short as one year) and generally follows a period of particularly heavy ethanol intake.

\(^{1}\) 1 typical drink = 13.6 g of ethanol.
Outcome in ALD patients is largely dependent on whether they continue to consume alcohol. Alcoholic steatosis is very rarely fatal (Morgan et al., 1978) and usually reverses fully within approximately four weeks of abstinence (Hall, 1987). In many cases AH is also reversible with abstinence, however in severe cases liver damage often increases in the first 2-4 weeks following cessation of drinking (Thompson et al., 1991). AH patients that continue to consume ethanol may suffer repeated and potentially lethal episodes of acute liver disease or may progress to cirrhosis. After initial diagnosis, the short-term mortality rate associated with acute AH has been estimated at 20% to 60% depending on the initial severity (Bird and Williams, 1990). In those that survive, roughly half progress to cirrhosis within 10 years (Sorenson et al., 1984). Alcoholic cirrhosis has a four year mortality rate of over 50% (Chedid et al., 1991; Powell and Klatskin, 1968; Finlayson, 1993).

The most common causes of death among ALD patients are liver failure (27-51%), variceal bleeding (9-47%), hepatocellular carcinoma (5-16%), infections (3-17%) and renal failure (1-8%) (Saunders and Latt, 1993). The risk of mortality is increased in patients with bilirubin levels above 15 mg/dL, abnormal coagulability, ascites or any degree of hepatic encephalopathy (Chedid et al., 1986; Maddrey et al., 1978; Carithers et al., 1989).

1.2 Mechanisms of Ethanol Hepatotoxicity

ALD involves aberrations in numerous biochemical pathways and it is often difficult to distinguish experimentally between causative and incidental factors. Considerable progress has been made in recent years in identifying several of the mechanisms by which ethanol exerts its toxic effects on the liver, however overwhelming evidence to support any one mechanism is still lacking and it is more likely that multiple mechanisms contribute to the pathogenesis of ALD. The observation that ALD only develops in a fraction of alcoholics who consume excessive levels of
ethanol is consistent with the multifactorial nature of the disease and suggests that individual
 genetic and environmentally-related factors play a role in determining susceptibility to ALD. For
 example, it is clear that women are at greater risk than men for developing ALD and do so after a
 shorter duration of exposure (Morgan and Sherlock, 1977) and at lower doses (Tuyns and
 Pequignot, 1984). Genetic polymorphisms in ethanol metabolizing enzymes have been reported
 recently which may account to some extent for both the predisposition to excessive alcohol
 consumption and/or the susceptibility to developing ALD in certain individuals. Environmental
 factors such as exposure to certain contaminants and drugs may also play a role in the etiology of
 ALD.

 The present studies focused primarily on three general mechanisms previously implicated
 in the pathogenesis of ALD: (a) the production of free radicals and reactive oxygen species (b)
 hepatic oxygen depletion and liver hypoxia; and (c) the formation of acetaldehyde, a toxic
 byproduct of ethanol metabolism. A review of these mechanisms, which are relevant with respect
 to both the etiology of ALD and the possible mode of action of PTU, is presented in the following
 section along with other processes currently implicated in the development of ALD.

 1.2.1 Free Radicals and Reactive Oxygen Species

 1.2.1.1 Free Radical Biochemistry: The reduction of molecular oxygen to water by
 the addition of four electrons is the major source of energy for most aerobic organisms. The
 partial reduction of dioxygen, which occurs in a variety of normal and pathologic situations,
 results in the formation of oxygen free radicals. Electrons in atoms and molecules occupy regions
 of space termed orbitals, each of which holds a maximum of two electrons. A free radical is
 defined as any chemical species capable of independent existence that contains one or more
 unpaired electrons, that is, an electron that is alone in an orbital. Since electrons are more stable
when paired together in orbitals, many free radicals are highly reactive because of the tendency of the unpaired electron to pair with another electron in an adjacent molecule. Free radicals, because of their high degree of reactivity, are capable of interacting with a wide variety of important biological molecules and causing pathological alterations in the structure and function of cells. Considerable evidence has implicated free radical-mediated damage as an important factor in the etiology of many diseases, including alcoholic liver disease.

The formation of free radicals can occur by several types of synthetic reactions. Homolytic cleavage of a peptide bond in a non-radical, results in the formation of two free radical fragments with each retaining one of the paired electrons.

\[ X:Y \rightarrow X^- + Y^- \quad \text{(homolytic fission)} \]

Electron transfer reactions, that is the addition or loss of a single electron from a non-radical, also results in the formation of free radicals.

\[ A + e^- \rightarrow A^- \quad \text{(electron addition)} \]

Electron transfer is a far more common process in biological systems than is homolytic fission, which generally requires high energy input from either high temperatures, ultraviolet light or ionizing radiation (Cheesman and Slater, 1993).

Arguably the most important radicals in biological systems are radical derivatives of oxygen. Reduction of oxygen by transfer to it of a single electron produces the superoxide anion free radical (superoxide: \( O_2^- \))

\[ O_2 + e^- \rightarrow O_2^- \]

A two electron reduction of oxygen yields hydrogen peroxide:

\[ O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \]

Hydrogen peroxide is generated in biological systems \textit{via} the production of superoxide, that is two superoxide molecules can react together to form hydrogen peroxide and oxygen:
2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad \text{(dismutation)}

This reaction, referred to as dismutation, takes place spontaneously at a very slow rate or can be catalyzed by the enzyme superoxide dismutase (SOD: EC 1.15.1.1) (Fridovich, 1989). Hydrogen peroxide, although not a free radical, is classified as a reactive oxygen species (ROS), a term that includes not only oxygen free radicals but also non-radical oxygen derivatives that are involved in oxygen radical production or have oxidizing capacity similar to that of many free radicals (Halliwell, 1994).

The importance of hydrogen peroxide in free radical biochemistry is based mainly on its ability to break down and generate a highly reactive and damaging free radical, the hydroxyl radical (OH·).

\[
O_2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2
\]

This spontaneous reaction, referred to as the Fenton reaction, is not likely to occur to a great extent in biological systems due to the low steady state concentration of the reactants. The rate of this reaction is however accelerated in the presence of a transition metal catalyst such as iron or copper (Halliwell and Gutteridge, 1990).

\[
O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}
\]

\[
H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^- + Fe^{3+}
\]

Net: \quad O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^-

In the above reaction, commonly known as the iron catalyzed Haber-Weiss reaction (Haber and Weiss, 1934), superoxide acts as a reductant of ferric iron (Fe^{3+}) to its ferrous form (Fe^{2+}). These reduced metal ions are much more efficient Haber-Weiss catalysts than their oxidized counterparts. Since most metal ions exist \textit{in vivo} in the oxidized state (Halliwell and Gutteridge, 1984), this reaction represents an important mechanism for the generation of hydroxyl radicals in biological systems.
Superoxide and hydrogen peroxide are not especially reactive nor are they thought to cause significant damage directly (Fridovich, 1978; Halliwell and Gutteridge, 1989). Rather their pathological significance appears to be based on their ability to generate OH·. In contrast, OH· is extremely unstable and will react with most biological molecules at diffusion controlled rates. Therefore OH· will not migrate a significant distance within a cell before reacting and has an extremely short half-life, but is capable of causing considerable damage within a small radius of its site of production (Pryor, 1986).

Hydroxyl radicals may also lead to the formation of other secondary carbon-centered radicals arising from the reaction of the oxidizing radical (OH·) with other biological molecules such as lipids, nucleic acids, carbohydrates or proteins (R·). These in turn react very rapidly to form the corresponding peroxyl radicals (ROO·).

The reaction of OH· with ethanol can lead to the formation of α-1-hydroxyethyl radicals, carbon-centered radicals which are considerably more stable and have longer half-lives than hydroxyl radicals. As such they may exert effects on biological systems remote from the site of their production and, as discussed in the following sections, may play an especially important role in the etiology of ALD.

1.2.1.2 Biological Sources and Targets of Free Radicals: Free radicals can be generated accidentally or deliberately by a variety of animal cell types. Under normal circumstances, the major source of free radicals in cells is electron leakage from electron transport chains such as those in the mitochondria and endoplasmic reticulum, to molecular oxygen, generating superoxide. Another source of superoxide is from the autoxidation of certain compounds such as ascorbic acid, thiols, adrenaline and flavin enzymes such as NADPH cytochrome P450 reductase. Superoxide can also be generated directly by a variety of enzymes
with oxidase activity, including NADPH oxidase, xanthine oxidase, aldehyde oxidase and cyclooxygenase. Activated neutrophils and other phagocytic cells deliberately generate superoxide, hypochlorous acid, hydrogen peroxide and hydroxyl radicals as part of their bactericidal role. Although free radicals are usually produced only at the interface of the phagocyte plasma membrane and bacterium, some accidental release of superoxide, hydrogen peroxide and other reactive oxygen species is likely (Smith, 1994; Kehrer, 1993).

All the major classes of biomolecules may be attacked by free radicals but lipids appear to be the most susceptible. Cells are rich in polyunsaturated fatty acids (PUFA) which are readily attacked by oxidizing radicals. The oxidative destruction of PUFA known as lipid peroxidation is particularly damaging because it is a self-perpetuating chain-reaction. Oxidation of PUFA generates a fatty acid radical (L·) that rapidly combines with oxygen to produce a fatty acid peroxyl radical (LOO·), which can in turn produce lipid radicals, hydroperoxides (LOOH) and additional peroxyl radicals. The breakdown of lipid hydroperoxides yields peroxyl and alkyl lipid radicals and aldehydes. The lipid peroxidation reaction sequence can be summarized as follows:

\[
\begin{align*}
LH + R^* & \rightarrow L^* + RH \\
L^* + O_2 & \rightarrow LOO^* \\
LOO^* + L^*H & \rightarrow LOOH + L^{*} \\
LOOH & \rightarrow LO^*, LOO^*, \text{aldehydes}
\end{align*}
\]

Lipid peroxidation plays a potentially important role in radical-mediated tissue injury since it is (a) a very likely occurrence given the availability and susceptibility of PUFA in cell membranes and (b) it is a very destructive reaction that can directly damage the structure of membranes and indirectly damage cells by the production of reactive aldehydes.

Proteins and nucleic acids, although not associated with the same damaging chain reactions as lipids, can also serve as targets for free radicals. Reactive oxygen species can cause
metal-catalyzed protein oxidation with introduction of carbonyl groups into the molecule, fragmentation or crosslinking. Sufhhydryl groups (e.g. cytseine and methionine) and amino groups (e.g. lysine, histidine) are common targets for oxidation by free radicals. Oxidation of enzymes may lead to a loss of activity and enhanced susceptibility to degradation by endogenous proteinases (Stadtman and Oliver, 1991).

DNA is readily attacked by oxidizing free radicals, an effect that accounts for the well known alterations in DNA associated with ionizing radiation in biological organisms. When hydroxyl radical is generated adjacent to DNA it attacks both the deoxyribose sugar and the purine and pyrimidine bases (Halliwell, 1994). The interaction of free radicals with DNA can result in strand breaks and cross-linkage (Sies, 1986) and even with a very high level of efficiency of DNA repair, sufficient damage may accumulate over a lifetime to lead to mutations and ultimately tumor formation.

It should be pointed out that free radicals produced in biological systems are not always destructive, and often play an essential role in normal physiological functions. For example, the nitrogen-centered free radical nitric oxide (NO), formed by endothelial cells and neutrophils from the amino acid L-arginine, is a vasodilator and is thought to play an important role in maintaining vascular tone (Moncada and Higgs, 1993).

1.2.1.3 Oxidative Stress and Antioxidant Defences: A state of equilibrium normally exists in vivo between the production of ROS and their deactivation by antioxidant systems, however a state of oxidative stress is associated with “a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage” (Sies, 1991). The production of free radicals is essential to normal metabolism but they can be destructive if their toxicity is not controlled. The accidental production of free radicals in biological systems is kept to a minimum.
by the high efficiency of most enzyme-mediated electron transfer reactions. In addition, cells have developed a comprehensive array of antioxidant defences to prevent free radical formation or limit their damaging effects. These include enzymes to decompose peroxides, proteins to sequester transition metal ions and a range of compounds to scavenge free radicals. The extensive measures taken by cells to minimize the toxicity of free radicals is often interpreted as an indirect indication that such reactions are detrimental to biological systems.

The first line of defence against superoxide is the enzyme SOD, which catalyzes the diffusion-limited dismutation of superoxide to hydrogen peroxide and oxygen (see 1.2.1.1). In the presence of SOD the rate of dismutation of superoxide is accelerated by roughly four orders of magnitude and intracellular levels of superoxide are maintained at approximately $10^{-11}$ M during normal metabolism (Morris et al., 1995). In mammalian cells three forms of SOD exist, a mitochondrial form containing manganese at its active site (MnSOD), a cytosolic form which contains copper and zinc at its active site (Cu/ZnSOD), and a copper-containing form located in the extracellular space (EC-SOD). Because SOD enzymes generate H$_2$O$_2$, they work in concert with H$_2$O$_2$ removing enzymes.

Hydrogen peroxide is decomposed by catalase, a heme-containing enzyme found in peroxisomes, and glutathione peroxidase (GSHPX) a selenium-containing enzyme found in the cytosol of most cells.

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$  \hspace{1cm} \text{(catalase)}

$$\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow 2\text{H}_2\text{O} + \text{GSSG}$$  \hspace{1cm} \text{(GSHPX)}

In addition to serving as a substrate for GSHPX, glutathione can also scavenge various free radicals directly (Chance et al 1979).

In the cell membrane $\alpha$-tocopherol, a major member of the vitamin E family, is a lipid-soluble chain-breaking antioxidant that functions to intercept lipid peroxyl radicals (LOO$^-$) and so
to terminate lipid peroxidation chain reactions:

\[ \text{LOO}^* + \alpha\text{-tocopherol-OH} \rightarrow \text{LOOH} + \alpha\text{-tocopherol-O}^* \]

The resulting tocopherol radical is relatively stable and generally insufficiently reactive to initiate lipid peroxidation itself.

Ascorbic acid (vitamin C) is an important water soluble antioxidant both within cells and in the plasma and may also serve to regenerate \( \alpha\text{-tocopherol} \) from the \( \alpha\text{-tocopherol-O}^* \) radical. The reaction between ascorbic acid and free radicals generates the semidehydroascorbate radical, which like the tocopherol radical is weakly reactive (Bielski et al., 1975).

\( \beta\text{-carotene} \), a precursor of vitamin A, acts as an antioxidant by reacting with singlet oxygen and peroxyl radicals, and therefore interferes with lipid peroxidation (Bjorneboe and Bjorneboe, 1993).

One of the major antioxidant defence mechanisms in mammals is the sequestration of transition metal ions into forms incapable of stimulating free radical reactions (Halliwell and Gutteridge, 1984). Transferrin, ferritin and lactoferrin are metal binding proteins that serve to limit the availability of catalytic metals in the extracellular fluids of the human body. Under certain circumstances however, such as iron overload diseases and after severe injury where metal ions can be released as a result of cell destruction, metal binding proteins may not be able to fully prevent metal-catalyzed free radical formation (Halliwell and Gutteridge, 1984).

1.2.1.4 Ethanol and Oxidative Stress: There is considerable experimental evidence to support a pathogenic role for oxidative stress in ethanol-induced liver injury. A variety of mechanisms have been proposed by which ethanol could increase the production of reactive oxygen species (ROS) or impair antioxidant defenses in the liver. Enhanced production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) occurs as a consequence of chronic ethanol exposure in a variety of cell types and
subcellular organelles. These compounds may contribute to liver injury by forming the highly toxic radical OH via the iron-catalyzed Haber-Weiss reaction. Neutrophils and the liver microsomal enzymes CYP2E1 and CYP-reductase, appear to play a pivotal role in ethanol-induced oxidative stress and are the focus of the present studies on the therapeutic mechanisms of PTU. The ability of these systems to generate free radicals is discussed in greater detail in sections 1.2.6 and 1.2.7. The following section provides an overview of other evidence demonstrating that oxidative stress accompanies ethanol-induced liver injury.

**Lipid Peroxidation:** The hypothesis that ethanol-induced liver injury could be linked to oxidative stress was based on early observations by Di Luzio and colleagues that lipid peroxidation in rat liver homogenates, measured from the formation of lipoperoxidation byproducts such as malondialdehyde, is stimulated in the presence of ethanol and that ethanol-induced fatty liver could be prevented by antioxidants (Di Luzio, 1963; Di Luzio and Hartman, 1967). Several subsequent studies have confirmed that ethanol both acutely and chronically can promote lipid peroxidation (Dianzini, 1985; Videla et al., 1982; Tsukamoto et al., 1986; Morimoto et al., 1993). While the ability of ethanol to enhance lipid peroxidation is now generally accepted, the pathological significance of this event in liver injury remains unclear. The formation of aldehydic peroxidation products (e.g. malondialdehyde and 4-hydroxynonenal), occurring secondary to lipid peroxidation, have recently been implicated as a potential mechanism for the damage. These products can stimulate the formation of collagen by lipocytes and myofibroblasts and may play a role in the development of alcoholic fibrosis (Chojkier et al., 1989).

**Formation of α-hydroxyethyl radicals:** Hydroxyethyl radicals (HER), formed by the reaction of hydroxyl radicals with ethanol, are generated during the metabolism of ethanol in
isolated human neutrophils (Ramos et al., 1992), rat liver microsomes (Reinke et al., 1990; Reinke et al., 1994a; Moncada et al., 1994), and in the intact deer mouse (Knecht et al., 1990) and rat (Reinke et al., 1991; Moore et al., 1995; Knecht et al., 1995). In vitro, HER bind covalently with proteins, such as albumin, to form stable HER-protein adducts. Immunization of animals with HER-protein adducts generated in vitro, results in the formation of antibodies that recognize epitopes on the ethylated proteins (Moncada et al., 1994). Immunoglobulins (IgG and IgA) that recognize HER-modified proteins are detectable in the serum of alcoholics with cirrhosis but not in the serum of healthy controls or non-alcoholics with cirrhosis (Clot et al., 1995; 1996). HER-protein adducts may cause liver injury by activating complement or by initiating antibody dependent cell-mediated cytotoxicity (Roit et al., 1989), in which antigen-antibody recognition leads to neutrophil activation and degranulation. Neutrophils can promote liver injury through the release of reactive oxygen species and proteolytic enzymes (see 1.2.8).

Nutrient Status and Endogenous Antioxidants: Until the last two decades, primary malnutrition due to dietary deficiencies was considered the main cause of liver disease in the alcoholic. In 1975, Lieber and associates demonstrated the ability of ethanol to produce liver injury in baboons receiving nutritionally balanced diets and it is now accepted that ethanol per se can exert hepatotoxic effects independently of nutritional deficiencies. Nonetheless, a number of dietary factors and nutrient imbalances have been identified which may act synergistically with the direct toxicity of ethanol to produce liver injury.

Protein-calorie malnutrition and other nutrient deficiencies are common in ALD and may be caused not only by impaired intake but also by malabsorption and increased breakdown of nutrients, particularly in the presence of liver disease (Mendenhall et al., 1984; Marsano and McClain, 1991). Since alcohol is rich in energy (7.1 kcal/g), chronic alcohol consumption leads to
primary malnutrition by displacing other nutrients in the diet (Lieber, 1994a). In addition, gastrointestinal disturbances caused by liver disease in the alcoholic (i.e., nausea, vomiting and anorexia) may also contribute to deficient nutrient intake (Achord, 1987).

Deficiencies of antioxidant vitamins and cofactors in the blood and liver of alcoholics have been frequently observed. The hepatic levels of selenium and zinc, which serve as cofactors of the antioxidant enzymes GSH peroxidase and CuZn-SOD respectively, are lowered following acute ethanol intake (Houze et al., 1991). Alcoholics show decreases in the plasma levels of ascorbate (Bonjour, 1979), β-carotene, and α-tocopherol and in patients with ALD, lowered levels of β-carotene and α-tocopherol are associated with more severe liver injury (Ward and Peters, 1992). In rats, chronic ethanol feeding in combination with a diet low in α-tocopherol results in an increase in hepatic lipid peroxidation (Kawase et al., 1989).

Ethanol causes a decrease in hepatic GSH levels, particularly in the mitochondria (Fernandez-Checa et al., 1987). However, the decrease may be associated with increased efflux into the circulation or a reduced rate of synthesis, rather than depletion as result of oxidative stress (Spiesky et al., 1985). A decrease in the levels of glutathione peroxidase (GSHPX) in the liver following chronic ethanol treatment has also been reported by several investigators (Schisler and Singh, 1989; Rouach et al., 1997), however this may be accompanied by an increase in the levels of GSHPX mRNA (Nanji et al., 1995b).

Several studies have shown that total dietary fat intake and the type of fat consumed are important determinants of ethanol-induced liver injury. The diets of many alcoholics are such that the majority of calories (approximately 35%) are derived from fat (Simpson and Peters, 1993; Lieber and DeCarli, 1989). In rats receiving chronic ethanol, an increase in the dietary fat intake from 6% to 25% of total calories results in significant enhancement of ethanol-induced liver injury (Tsukamoto et al., 1986). The feeding of liquid diets containing ethanol and fish oil, which is rich
in ω-3 polyunsaturated fatty acids, produces liver injury in rats that is similar in appearance to human alcoholic hepatitis (Nanji et al., 1994a). In comparison diets containing ethanol and fat supplied as corn oil, which is less rich in ω-3 polyunsaturated fatty acids, results in less severe liver injury (Nanji et al., 1994a; French, 1993). In addition, the greater severity of liver injury seen in rats fed ethanol and polyunsaturated fat is associated with higher levels of lipid peroxidation (Nanji et al., 1994a). In countries where the consumption of polyunsaturated fats is relatively high, the frequency of alcoholic cirrhosis is greater than would be predicted by *per capita* ethanol consumption (Nanji and French, 1986). A therapeutic regimen consisting of a diet enriched in saturated fatty acids (palm oil) was shown to reverse the pathological changes induced by chronic ethanol, while treatment with a diet enriched in ω-3 polyunsaturated fatty acids (fish oil) did not lessen the severity of ethanol-induced liver injury (Nanji et al., 1995a). Further studies have shown that the extent of ethanol-induced liver injury is influenced by the linoleic acid content of the diet. Rats fed ethanol in diets containing corn oil, which is rich in linoleic acid (56.6%) showed more severe liver injury than those receiving diets with ethanol and lard (2.5%) or beef tallow (0.7%) (Nanji et al., 1989).

Increased levels of iron in the liver following chronic ethanol consumption have been reported in rats (Shaw 1989) and in human alcoholics with ALD (Williams *et al*., 1967; Zimmerman *et al*., 1961). Chronic ethanol consumption increases the absorption of iron from the gastrointestinal tract (Irving *et al*., 1988). In addition, the high iron content of certain alcoholic beverages may also contribute to elevations in tissue iron levels (Barry, 1973; Sherlock, 1981; Baker, 1986). *In vitro*, superoxide has been shown to mobilize iron bound by ferritin (Biemond *et al*., 1984). The ability of chronic ethanol to increase the formation of superoxide in the liver may result in an increase in the availability of catalytically active iron, which in turn could promote liver injury through the formation of hydroxyl radicals by the iron-catalyzed Haber-Weiss reaction.
In humans, iron storage disorders (i.e., hereditary hemochromatosis) are characterized by substantial elevations in hepatic iron content (Bassett et al., 1986). These patients have elevated serum levels of lipid peroxidation products (Young et al., 1994), as well as an increased incidence of cirrhosis (Bassett et al., 1986) and hepatocellular carcinoma (Niederau et al., 1985). In rats, iron supplemented diets have been shown to greatly increase the severity and rate of development of ethanol-induced liver injury (Tsukamoto et al., 1992; Stal et al., 1996).

Mitochondrial Dysfunction: Chronic ethanol consumption is associated with numerous defects in the mitochondrial electron transport chain that may result in enhanced production of ROS (Cederbaum and Rubin, 1975; French, 1992). Reduction of oxygen by the mitochondrial respiratory chain is normally tightly coupled via cytochrome oxidase to four-electron reduction of water. Inhibition of the respiratory chain and uncoupled respiration increases production of superoxide and hydrogen peroxide (Forman and Boveris, 1982). Damage to the mitochondrial respiratory chain as a consequence of chronic ethanol treatment (Cunningham et al., 1990; Thayer and Rubin, 1979; Forman and Boveris, 1982) can promote autoxidation of reduced electron carriers to yield ROS. Acute administration of ethanol in vivo enhances superoxide production in rat liver submitochondrial particles (Sinaceur et al., 1985). Mitochondria isolated from rats following chronic ethanol treatment show an approximately 50-100% increase in the rate of NADH-dependent hydroxyl radical formation and a two- to three-fold induction in mitochondrial lipid peroxidation (Kukielka et al., 1994)

Xanthine Oxidase and Aldehyde Oxidase: Acute ethanol treatment causes the conversion of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) which can generate superoxide radicals (Sulatos et al., 1988). Acetaldehyde derived from ethanol oxidation can serve as a
substrate for xanthine oxidase and generate superoxide, however the Km of XO (>30 mM) (Fridovich, 1966) is well in excess of acetaldehyde concentrations typically found in the liver (Erickson, 1987). Acetaldehyde may also lead to superoxide generation through aldehyde oxidase, a molybdoflavin enzyme closely related to XO but with much greater affinity for acetaldehyde (Shaw and Jayatilleke, 1990).

1.2.2 Oxygen Depletion and Liver Hypoxia

As blood flows through the liver lobule, oxygen and nutrients are extracted and metabolites generated, establishing gradients between the portal vessels and central vein. An oxygen concentration gradient exists within the liver lobule characterized by decreasing oxygen saturation from portal to central regions (Thurman et al., 1984b). Following an acute dose of ethanol, oxygen consumption in the perfused liver is increased by 50-80% as compared to controls. Chronic ethanol administration in rats has been shown to increase hepatic oxygen consumption by 30-60%, as measured in the perfused liver in situ (Britton et al., 1984; Rachamin et al., 1985), and by 40-160% in vivo (Carmichael et al., 1993; Tsukamoto and Xi, 1989). A consequence of the alcohol-induced increase in oxygen uptake (hypermetabolic state) may be a steeper oxygen gradient within the liver lobule. The existence of a hypermetabolic state in human alcoholics with either hepatitis or cirrhosis has been observed (Shanbhoque et al., 1987; John et al., 1989). Israel et al. (1975a) postulated that ethanol-induced liver injury is caused by centrilobular hypoxia resulting from such an exaggerated oxygen gradient - i.e., the oxygen gradient becomes so steep that centrilobular regions, those regions furthest removed from the oxygen supply, are not supplied sufficient oxygen. In the rat, the hypermetabolic state is compensated for by an increase in portal blood flow and oxygen delivery when ethanol is present in the blood, so as to prevent oxygen depletion (Orrego et al., 1988; Carmichael et al., 1993).
However after ethanol withdrawal the compensatory increase in hepatic blood flow no longer occurs, while the ethanol-induced hypermetabolic state continues, generating a state of relative oxygen insufficiency. In support of the hypoxia theory is the observation that centrilobular hepatocyte necrosis is induced by hypoxia or experimental anemia in chronic ethanol-fed rats (Israel et al., 1975; French et al., 1984). Recent studies by Arteel et al. (1996; 1997) using 2-nitroimidazole as markers of hypoxia in chronic ethanol-treated rats, provide further evidence that hypoxia is involved in mechanisms of early alcoholic liver injury.

Kupffer cells, resident macrophages of the liver, may play an important role in mediating the ethanol-induced hypermetabolic state. Increased levels of circulating bacterial endotoxins are associated with alcoholic liver disease (Fukui et al., 1991; Nanji et al., 1993) and serve as a potent stimulus for the release of inflammatory mediators by Kupffer cells (Nolan, 1981). It has been postulated that Kupffer cells are activated by ethanol, through increased circulating endotoxin levels, to release PGE\(_2\) which stimulates oxygen consumption in hepatic parenchymal cells (Qu et al., 1996). Inactivation of Kupffer cells, by treatment with the selective Kupffer cell toxin gadolinium chloride (Koudstaal et al., 1991), prevents both the ethanol-induced increase in hepatic oxygen consumption (Bradford et al., 1993) and ethanol-induced liver injury (Adachi et al., 1994; Koop et al., 1997).

Ethanol-mediated induction of cytochrome P4502E1, a P450 isozyme present in liver microsomes, may also contribute to hepatic oxygen depletion (see 1.2.6). The ability of PTU to prevent the ethanol-induced enhancement of oxygen consumption in liver microsomes is a mechanism which has been examined in the present investigations (see Study 1).
1.2.3 Acetaldehyde

Acetaldehyde is normally formed as a metabolic byproduct of ethanol oxidation. During ethanol metabolism the levels of acetaldehyde in vivo are extremely low, due to the efficiency of enzymatic reactions involved in the breakdown of acetaldehyde. However, chronic ethanol consumption results in a significant reduction in the mitochondrial elimination of acetaldehyde (Hasamura, 1975; Baraona et al., 1987), and is associated with a significant increase in acetaldehyde levels in the venous blood, indicative of elevated tissue levels (Roine et al., 1993; Lucas et al., 1986).

Excess formation of acetaldehyde has been implicated as an important initiating factor in the pathogenesis of ALD. Acetaldehyde is a highly reactive molecule that may form covalent bonds with endogenous enzymes and other proteins. Lysine residues and sulfhydryl groups are particularly susceptible targets and binding to albumin, hemoglobin, tubulin, a 37 kD liver protein and calmodulin as well as certain enzymes including ribonuclease, has been reported (Jennet et al., 1990; Lin and Lumeng, 1989). Acetaldehyde-modified proteins are present in the centrilobular area of the liver in rats fed ethanol chronically and in the liver of humans with ALD (Niemela et al., 1994). By mechanisms similar to those described for HER-protein adducts (see 1.3.1.4), acetaldehyde-protein adducts may contribute to liver injury by triggering an immune reaction. Circulating antibodies to acetaldehyde modified proteins have been detected in patients with alcoholic hepatitis but not patients with fatty liver or cirrhosis (Niemela et al., 1987; Koskinas et al., 1992). In guinea pigs, immunization with acetaldehyde-protein adducts generated in vitro, produced necrosis and inflammation similar to that of human alcoholic hepatitis, following the chronic administration of ethanol (Yokayama et al., 1993).

The lysine residues and sulfhydryl groups of α-tubulin, a protein involved in the formation of hepatic microtubules, are modified by acetaldehyde. Binding of acetaldehyde to α-tubulin
prevents its polymerization and damages the microtubular structure of cells leading to impaired protein secretion and accumulation of secretory proteins such as albumin and transferrin (Matsuda et al., 1979; Pignon et al., 1987). It has been suggested that the swelling of hepatocytes and sinusoidal compression characteristic of ALD (Blendis et al., 1982) may be due to an osmotically driven increase of intracellular water and ions, caused by the retention of proteins within hepatocytes (Lieber, 1988). Studies by Israel et al. (1982) suggest that it is more likely that intracellular accumulation of potassium ions, rather than the retention of proteins, contributes to the osmotic increase in intracellular water and hepatocyte volume.

Acetaldehyde has also been shown to activate rat hepatic stellate cells (formerly known as Ito cells or lipocytes) to increase transcription of the genes for procollagen type I and fibronectin and may therefore play a role in the pathogenesis of fibrosis (Casini et al., 1991).

Given the importance of acetaldehyde in the toxicity of ethanol, considerable research interest has focused on ethanol metabolizing enzymes such as the alcohol and aldehyde dehydrogenases and cytochrome P4502E1 and their possible contribution to the development of ALD. Additional pathways for the metabolism of ethanol do exist in vivo, such as H2O2-mediated oxidation by catalase (Chance et al., 1979) and non-oxidative metabolism to fatty acid ethyl esters (Laposta and Lang, 1986). However as the contribution of these reactions to the overall elimination of ethanol and their significance in liver injury appears to be minor (Koop, 1989), they will not be discussed further.

1.2.4 Alcohol Dehydrogenase

Alcohol dehydrogenase (EC 1.1.1.1) is a soluble zinc metalloenzyme found in high concentrations in the liver and plays a major role in ethanol metabolism, accounting for roughly
80-90% of ethanol oxidation in the body (Li, 1977). ADH catalyzes ethanol oxidation according to the following reaction:

\[ \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \rightarrow \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ \]

In this reaction acetaldehyde is produced and hydrogen is transferred from ethanol to the cofactor NAD\(^+\), which is converted to its reduced form NADH.

ADH isoforms have been detected in many mammalian tissues, although most activity is detected in the liver with smaller amounts found in stomach, intestine and kidney (Li, 1977; Smith et al., 1971). These exist in multiple molecular forms which arise from the association of eight different types of subunits - \(\alpha\), \(\beta_1\), \(\beta_2\), \(\beta_3\), \(\gamma_1\), \(\gamma_2\), \(\pi\) and \(\chi\) - into active homodimeric and heterodimeric molecules. The various subunits are coded at six gene loci, designated ADH1 through ADH6 (Bosron et al., 1993; Yasunami et al., 1991). The various isoforms of ADH in humans are assigned to five classes (Pares et al., 1992), several of which can contribute to the oxidation of ethanol \textit{in vivo}. The class I enzymes, the common liver ADHs, consist of dimers of \(\alpha\), \(\beta\) and \(\gamma\) subunits, encoded for by the ADH1, ADH2 and ADH3 genes respectively. They have a high affinity for ethanol, with a \(K_m\) of 0.1 to 1.0 mM, and they are very sensitive to inhibition by pyrazole. A class II ADH, designated \(\pi\)-ADH, is expressed in human liver (Li et al., 1977) and to a lesser extent in the intestine (Smith et al., 1972), and is encoded for by the \(ADH4\) gene. The liver enzyme activity is less than 10% of that of the class I ADH at low ethanol concentrations but may become important at higher ethanol concentrations (Li et al., 1992). The enzyme has a relatively high \(K_m\) for ethanol (34 mM) and is relatively insensitive to inhibition by pyrazole. Class III ADH, is coded for by the \(ADH5\) gene and consists of a dimer of \(\chi\) subunits. The enzyme has a very low affinity for ethanol (\(K_m > 1\) M) and is therefore not thought to contribute to ethanol metabolism \textit{in vivo}. The class IV ADH designated as \(\sigma\)-ADH or \(\mu\)-ADH, has a similar \(K_m\) for ethanol (41 mM) as the class II ADH (Moreno and Pares, 1991). The enzyme is expressed
predominantly in the stomach and may contribute to gastric ethanol metabolism. Class V ADH consists of a single recently cloned gene (ADH6) which is expressed in liver and stomach (Yasunami et al., 1991; Chen et al., 1991). The enzyme has a $K_m$ for ethanol of 28 mM and its role in the oxidation of ethanol is presently unknown.

In humans, ADH polymorphisms occur at two loci, ADH2 and ADH3, which encode the β and γ subunits (Burnell and Bosron, 1989). Because ADH is an important rate-limiting factor in ethanol metabolism there have been attempts to correlate ethanol elimination rates, and predisposition to alcoholism and alcoholic liver disease with ADH genotype. One of the allelic variants of ADH, designated ADH$_2^2$, is uncommon in American and European Caucasians but is found in up to 85% of the Oriental population (Agarawal et al., 1981; Bosron et al., 1988). The corresponding enzyme shows an increased rate of ethanol oxidation at high ethanol concentrations, as compared to the ADH$_2^1$ variant (Bosron et al., 1988). In individuals with the variant allele the metabolism of acetaldehyde by the enzyme acetaldehyde dehydrogenase may become the rate-limiting reaction and acetaldehyde may accumulate resulting in a disulfiram-like aversive reaction to ethanol (see below). Therefore individuals with the mutant gene may be less likely to become alcoholic or to develop ALD (Thomasson et al., 1991).

1.2.5 Aldehyde Dehydrogenase

Acetaldehyde produced from the oxidation of ethanol is further metabolized to acetate by aldehyde dehydrogenase (ALDH: EC 1.2.1.3), which is also associated with the conversion of NAD$^+$ to NADH:

$$
\text{CH}_3\text{CHO} + \text{NAD}^+ + \text{H}_2\text{O} \longrightarrow \text{CH}_3\text{COOH} + \text{NADH} + \text{H}^+
$$

ALDH enzymes are expressed in most cells of the body with highest activity found in the liver (Tipton et al., 1989). Of the three known classes of ALDH, the class II enzymes (ALDH$_1$ and
ALDH₂) are thought to play an important role in the elimination of acetaldehyde in vivo. ALDH₁, isolated from the hepatic cytosol, and ALDH₂, from the mitochondria, are homotetramers with a subunit molecular weight of 54 kD. Although both ALDH₁ and ALDH₂ have relatively high affinity for acetaldehyde (Kₘ = 17 μM and 0.2 μM respectively), the greater activity of ALDH₂ at low acetaldehyde concentrations suggest that it is the major pathway of ethanol oxidation in vivo (Tipton et al., 1989).

Inhibitors of ALDH, such as disulfiram (Antabuse ®) (Hald et al., 1948) and calcium carbimide (Temposil ®) (Bell, 1956), have been used for over 40 years in the aversion therapy of alcoholism. Inhibition of ALDH by these agents results in the accumulation of acetaldehyde when ethanol is consumed, which produces a number of unpleasant side-effects (flushing, hypotension, nausea, vomiting).

A clinically important genetic polymorphism in the ALDH₂ gene (ALDH₂³) is expressed in roughly 50% of the Oriental population (Ikawa et al., 1983; Goedde et al., 1992). The mutant allele codes for an inactive form of the enzyme and is closely associated with the “flushing response” in Orientals, producing a reaction similar to that of disulfiram. Circulating acetaldehyde levels can be more than twenty times higher (30-100 μM) in individuals who produce the inactive variant than in those who produce the active form of the enzyme (Mizoi et al., 1983; Eriksson, 1983; Agarawal and Goedde, 1987).

The reactions catalyzed by ADH and ALDH generate an excess of reducing equivalents as free NADH in the hepatic cytosol, primarily because the metabolic systems involved in the removal of NADH are not able to offset its accumulation fully. The alteration in the oxidative-reductive capacity of the liver, reflected by the increased ratio of NADH to NAD⁺, is thought to account for several hepatic and metabolic disorders observed in ALD (Lieber, 1994b).
1.2.6 Cytochrome P4502E1

In 1968 Lieber and DeCarli first described a microsomal ethanol oxidizing system (MEOS) in the liver which could be dissociated from ADH and catalase, the other two liver enzymes capable of metabolizing ethanol to acetaldehyde in vitro. In contrast to ADH the microsomal oxidation was more active with NADPH than with NAD⁺, had a neutral pH optimum and showed a relative insensitivity to the ADH inhibitors pyrazole and 4-methylpyrazole (Teschke et al., 1976; Lieber and DeCarli, 1970a). In addition to ethanol, the microsomal enzyme also catalyzed the oxidation of higher aliphatic alcohols such as butanol (Teschke et al., 1976), which are not substrates for catalase (Teschke et al., 1975). Chronic ethanol treatment in the rat was associated with an increase in the activity of MEOS (Lieber and DeCarli, 1970a). The induction of MEOS activity was shown to be associated mainly with the appearance of an ethanol-specific isozyme of the hemoprotein cytochrome P450 (Ohnishi and Lieber, 1977). The enzyme, later designated as CYP2E1 (EC 1.5.99)², has since been purified and characterized in several mammalian species including humans.

The polypeptide and cDNA sequences of CYP2E1 have been determined in humans and rats (Song et al., 1986), rabbits (Khani et al., 1987) and hamsters (Sakuma et al., 1994). The relative molecular weight of CYP2E1 is 51000 in the rabbit, 51500 in rats, 54000 in humans and 56000 in hamsters. Although they are slightly different in electrophoretic mobility and peptide maps, CYP2E1 has similar catalytic, spectral and immunochemical properties, as well as significant homology of amino-terminal sequences among the different species examined. A single gene codes for the CYP2E subfamily in all species studied to date, with the exception of rabbits

² Based on the P450 nomenclature standards of Nebert et al (1987), CYP2E1 replaces the former designations P450LM3A (rabbit), P450j (rat) and P450HLj (human). Although CYP2E1 has been identified as the principal enzyme regulating MEOS activity, other P450 isoforms may also be induced by ethanol (i.e. CYP2B1/2, 3A, 2C and 1A2- Sinclair et al., 1991; Ronis et al., 1990; Asai et al., 1996). Therefore the term MEOS is still used to refer to the overall capacity of microsomes to oxidize ethanol, rather than to the fraction of the activity that is catalyzed solely by CYP2E1 (see Lieber, 1988; Crabb and Lands, 1992).
which express CYP2E1 and a second subtype CYP2E2, which has similar catalytic activity but a
different pattern of ontological development from that of CYP2E1 (Porter et al., 1989). In
humans the CYP2E1 gene has been mapped to chromosome 10 (Umeno et al., 1988).

CYP2E1 is mainly found in the liver smooth endoplasmic reticulum but significant
amounts are also found in hepatic Kupffer cells (Koivisto et al., 1996) and in a variety of other
non-hepatic tissues including brain (Hansson et al., 1990), lung and kidney (Zerilli et al., 1995),
although the basal levels of CYP2E1 protein in these tissue are at least 10-20 times lower than
that of liver. CYP2E1 is also expressed on the exterior surface of the plasma membrane of rat
hepatocytes (Wu and Cederbaum, 1992). In the liver CYP2E1 is not expressed in newborns but
from 1 month to 1 year post-gestation, the protein content gradually increases and is accompanied
by the accumulation of CYP2E1 RNA, suggesting a transcriptional activation of the gene during
the late neonatal period (Song et al., 1986; Vieira et al., 1996).

Mutations of the CYP2E1 gene have been reported in the 5'-flanking region, TATA box
and in the GT-rich region in the 3'-flanking region, and may be associated with alterations in
enzyme activity and the stability of the corresponding mRNA in vivo (Ingelman-Sundberg et al.,
1993; Persson et al., 1993). Several polymorphisms of CYP2E1 have been identified using
restriction fragment length polymorphism (RFLP) analysis and the restriction enzymes Taq I, Dra
I, Rsa I and Pst I. The Taq I and Dra I RFLPs are two allele polymorphisms occurring within
intron 7 and intron 2 respectively (McBride et al., 1987; Uematsu et al., 1991). More recently,
two novel Rsa I and Pst I RFLPs in the 5'-flanking (i.e., regulatory) region of the human CYP2E1
gene have been detected (Watanabe et al., 1990; Hayashi et al., 1991). The predominant
homozygous allele, heterozygous allele and rare homozygous allele have been designated as type
A (c1/c1), type B (c1/c2) and type C (c2/c2), respectively (Watanabe et al., 1990). The type C
phenotype may be associated with increased expression of hepatic CYP2E1 protein (Hayashi et al., 1991).

CYP2E1 is not homogeneously expressed or distributed within the liver acinus. The expression of the enzyme, both constitutively and after induction, is restricted to the centrilobular region of the liver, and in particular to the three to four layers of hepatocytes most proximal to the central vein (Buhler et al., 1990; Ingelman-Sundberg et al., 1988). The concentration of CYP2E1 in liver microsomes is roughly 0.4 nmol/mg (Johansson et al., 1988) and it has been estimated that the concentration in perivenous hepatocytes can be as high as 50-100 μM following induction by chronic ethanol (Persson et al., 1990). Since the first purification of CYP2E1, the catalytic activity of the enzyme has been extensively studied. More than 50 substrates for CYP2E1 have been identified thus far (Table 1) (c.f. Terelius et al., 1992; Koop, 1992). In addition to ethanol, CYP2E1 selectively metabolizes a diverse range of other chemical compounds including aliphatic alcohols (e.g. butanol, isopropanol), organic solvents (e.g. CCl₄, acetone, toluene, benzene), therapeutic drugs and anesthetics (e.g. acetaminophen, ether and halothane) and nitrosamines (NDMA). Structurally these substrates have little in common except that they are generally of low molecular weight and are hydrophobic in nature. CYP2E1 participates in several different types of NADPH-dependent electron transfer including monooxygenation, reduction, oxidase and peroxidation reactions. The monooxygenation reaction catalyzed by CYP2E1 involves several steps: (a) substrate addition to ferric-CYP2E1 (b) transfer of the first electron from NADPH-cytochrome P450 reductase to CYP2E1 (c) oxygen binding to ferrous-CYP2E1 (d) addition of the second electron and (e) hydroxylation of substrate and release of product from the CYP2E1 molecule. In its action as a reductase CYP2E1 introduces one electron directly to the substrate. As a consequence radicals of the substrate are formed, as occurs for example from the reductive cleavage of carbon tetrachloride to form the trichloromethyl radical. Electrons donated by
Table 1: Substrates and Inducers of Cytochrome P4502E1

(Known inducers of CYP2E1 are italicized).

<table>
<thead>
<tr>
<th>Alcohols</th>
<th>Halogenated Hydrocarbons</th>
<th>Miscellaneous Compounds (cont.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,3-butanediol</td>
<td>carbon tetrachloride</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>n-butanol</td>
<td>chloroform</td>
<td>ethylcarbamate</td>
</tr>
<tr>
<td>ethanol</td>
<td>dichloromethane</td>
<td>methylazoxymethanol</td>
</tr>
<tr>
<td>glycerol</td>
<td>dichloropropane</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td>methanol</td>
<td>ethylene dichloride</td>
<td>N-nitrosodimethylamine</td>
</tr>
<tr>
<td>n-pentanol</td>
<td>methylene chloride</td>
<td>N-nitroso-2,6-dimethylmorpholine</td>
</tr>
<tr>
<td>1-propanol</td>
<td>trichloroethylene</td>
<td>N-nitrosomethylethylamine</td>
</tr>
<tr>
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<td>vinyl chloride</td>
<td>N-nitrosopyrrolineid</td>
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<td>Ketones</td>
<td>obesity</td>
</tr>
<tr>
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<td>acetone</td>
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<td>2-butanone</td>
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</tr>
<tr>
<td>m-xylene</td>
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<tr>
<td>Ethers</td>
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<td>alloxan-induced diabetes</td>
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</tr>
<tr>
<td>methyl t-butyl ether</td>
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<td>Halogenated Anesthetics</td>
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<td></td>
<td>clotrimazole</td>
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</tbody>
</table>

3 This list is compiled from previous experimental data as reviewed by Terelius et al., 1992 and Koop et al., 1992.
CYP2E1 may in the absence or presence of substrate, be accepted instead by dioxygen, reactions which constitute the oxidase activity of CYP2E1 (Ekstrom and Ingelman- Sundberg, 1989; Gorsky et al., 1984). Both two and four electron reduction pathways occur.

\[
\text{NADPH + H}^+ + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}_2\text{O}_2
\]

\[
2\text{NADPH} + 2\text{H}^+ + \text{O}_2 \rightarrow 2\text{NADP}^+ + 2\text{H}_2\text{O}
\]

The formation of hydrogen peroxide by CYP2E1, shown in the first reaction, is thought to result from autoxidation of CYP2E1, yielding superoxide which subsequently dismutates to \( \text{H}_2\text{O}_2 \) (Kuthan and Ullrich, 1982). However the direct release of \( \text{H}_2\text{O}_2 \) from the two-electron reduced enzyme is also possible (Gorsky et al., 1984; Guengerich, 1991).

In the oxidation of ethanol, CYP2E1 reduces molecular oxygen to water as ethanol is oxidized to acetaldehyde, according to the following reaction:

\[
\text{CH}_3\text{CH}_2\text{OH} + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{CH}_3\text{CHO} + \text{NADP}^+ + 2\text{H}_2\text{O}
\]

Acetaldehyde is also a substrate for CYP2E1, however the Km of the reaction is three to four orders of magnitude higher than that for ethanol (Terelius et al., 1991).

CYP2E1 has a high Km for ethanol (7-11 mM) (Teschke et al., 1974) as compared with ADH (< 2 mM) but may contribute substantially to ethanol metabolism at higher blood ethanol concentrations at which ADH is fully saturated. Unlike ADH, CYP2E1 is induced by chronic ethanol exposure and is therefore thought to be one of the mechanisms mediating the enhanced rate of ethanol metabolism (metabolic tolerance) associated with prolonged ethanol intake (Salaspuro and Lieber 1978).

Roughly a third of the known substrates of CYP2E1, including ethanol, also act as inducers of the enzyme (see Table 1). In addition to chemical inducers, certain pathophysiological states such as diabetes, obesity, fasting are also associated with induction of CYP2E1. Chronic ethanol consumption is associated with a 6-12 fold increase in the levels and activity of the
CYP2E1 in the rat (Badger et al., 1993a; Ronis et al., 1993). The molecular mechanism underlying CYP2E1 induction by ethanol may involve several mechanisms including enhanced enzyme synthesis, enzyme stabilization, increased levels of mRNA and increased efficiency of mRNA translation. Increased levels of CYP2E1 mRNA have been reported in rodents treated chronically with ethanol (Kubota et al., 1988; Diehl et al., 1991; Badger et al., 1993) and in human alcoholics (Takahashi et al., 1993). Different mechanisms may be responsible for CYP2E1 induction at high and low blood ethanol concentrations. At blood ethanol concentrations below 300 mg/dL the induction of CYP2E1 by ethanol in rats appears to be due to either increased efficiency of mRNA translation or decreased degradation of the enzyme rather than to an increase in CYP2E1 mRNA, while at blood ethanol concentration in excess of 300 mg/dL an increase in CYP2E1 mRNA also occurs (Badger et al., 1993a), via enhanced transcription of the CYP2E1 gene (Badger et al., 1993b). CYP2E1 substrates have the capability to protect the enzyme from degradation in hepatocytes (Eliasson et al., 1988). The mechanism involves inhibition of cAMP-dependent phosphorylation of CYP2E1 on Ser129 (Eliasson et al., 1990). In the absence of substrate, phosphorylation results in the loss of the heme group of CYP2E1 and the enzyme is rapidly degraded.

1.2.6.1 Role of CYP2E1 in Ethanol Hepatotoxicity: There is considerable direct and indirect evidence implicating a role for CYP2E1 in the pathogenesis of liver injury in ALD. Several mechanisms have been identified by which the induction of CYP2E1 by chronic ethanol intake may contribute to ethanol hepatotoxicity including: (a) increased formation of ROS; (b) hepatic oxygen depletion and (c) conversion of CYP2E1 substrates into toxic metabolites.

CYP2E1 is primarily expressed both constitutively and following induction, in zone III of the liver lobule around the central vein, the primary location where damage to hepatocytes occurs.
in ALD and in many other forms of toxin-induced liver injury. Like ethanol, which is oxidized to acetaldehyde, a variety of other substrates of CYP2E1 are metabolized to form compounds which are considerably more hepatotoxic and/or carcinogenic than the parent molecules (Raucy et al., 1993; Terelius et al., 1992). Many CYP2E1 substrates are potent hepatotoxins, producing liver injury that is (a) similar in appearance to that produced by ethanol, particularly with respect to the predominance of lesions in zone 3 of the liver acinus and (b) is potentiated by chronic ethanol exposure; these include acetaminophen (McJunkin et al., 1976; Seef et al., 1986), CCl₄ (Bruckner et al., 1986; Hasamura et al., 1974), N-nitrosodimethylamine (Anderson et al., 1986; Ma et al., 1991), benzene (Babani et al., 1991; Hetu et al., 1983), vinylidine chloride (Siegers et al., 1983), enflurane (Lewis et al., 1983; Tsutsumi et al., 1990) and halothane (Cousins et al., 1979; Takagi et al., 1983). Since the hepatotoxic and carcinogenic properties of many drugs, food additives and environmental contaminants may be heightened in the alcoholic it has been postulated that these agents may be a contributing factor in ALD.

Physiological conditions which result in the induction of CYP2E1, such as obesity and diabetes, are also associated with liver injury. Non-alcoholic steatohepatitis is a form of liver disease observed in obese individuals that closely resembles AH and can also progress to cirrhosis (Lee, 1989; Ludwig et al., 1980). Human diabetes and experimental diabetes induced in rats by the chemicals streptozotocin or alloxan, are also associated with liver pathologies similar to those observed in ALD (Falchuk et al., 1980; Vajdovich et al., 1993; Laguens et al., 1980; Levine et al., 1980).

Liver microsomes isolated from rats fed ethanol chronically have been shown to produce O₂⁻⁻ and H₂O₂ at elevated rates compared to pair-fed controls (Boveris et al., 1983; Lieber and DeCarli, 1970a; Thurman, 1973). CYP2E1 has the capacity to generate O₂⁻⁻ and H₂O₂ from the reduction of oxygen (Gorsky et al., 1984; Ingelman-Sundberg and Johansson, 1984). The
formation of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in liver microsomes exposed to a variety of inducing agents was shown to correlate well with the amount of CYP2E1 present in the microsomal membranes (Ekstrom and Ingelman-Sundberg, 1989). Chronic ethanol treatment results in an increase in the oxidase activity of CYP2E1 which may be associated with inefficient coupling between CYP2E1 and CYP-reductase, such that \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) are generated (Gorsky et al., 1984; Ekstrom and Ingelman-Sundberg, 1989). In the presence of transition metals this activity may be of importance for microsomal generation of \( \text{OH}^- \) by the Haber-Weiss reaction. Studies by Ingelman-Sundberg and Johansson (1984) using purified CYP2E1 and CYP-reductase in reconstituted microsomal membranes systems have shown that in the presence of low amounts of chelated iron (1 \( \mu \text{M} \)) CYP2E1 is an effective catalyst for the formation of \( \text{OH}^- \). Hydroxyl radicals generated by CYP2E1 may react with ethanol to form hydroxyethyl radicals (HER). The generation of HER has been demonstrated in liver microsomes incubated with ethanol and either NADPH (Alabano et al. 1988; Reinke et al., 1990) or NADH (Rao et al., 1996; Rashba-Step et al., 1993), and their formation is inhibited by about 50% in the presence of anti-CYP2E1 IgG (Alabano et al., 1990). The remaining activity may be attributable to the ability of CYP2E1 to directly catalyze the oxidation of ethanol to generate ethanol free radicals (Alabano et al., 1991) or to iron-dependent hydroxyl radical formation by CYP-reductase (see 1.2.7).

A further consequence of the ethanol-induced enhancement of CYP2E1 oxidase activity and ROS formation may be an increase in microsomal lipid peroxidation. Studies using reconstituted membrane systems containing CYP2E1 and CYP-reductase have demonstrated the production of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) and NADPH-dependent lipid peroxidation, which is inhibited to a large extent in the presence of anti-CYP2E1 IgG (Ekstrom and Ingelman-Sundberg, 1989). In rats, the variable effects of different dietary fats on ethanol-induced liver injury is closely correlated with the degree of CYP2E1 induction and the extent of lipid peroxidation. The feeding
of diets containing fish-oil results in a greater degree of lipid peroxidation, liver injury and induction of CYP2E1 as compared to diets containing corn-oil (Nanji et al., 1994a).

Several observations suggest that hepatic oxygen depletion may be another mechanism whereby CYP2E1 can contribute to ethanol-induced liver injury. Oxygen is consumed by the enzyme both in the oxidation of substrates and by oxygen reduction in the oxidase reactions catalyzed by CYP2E1, which occurs both in the presence and absence of substrates. CYP2E1 is expressed and induced primarily in zone III of the liver acinus (Buhler et al., 1991; Ingelman-Sundberg et al., 1988), the region where circulating oxygen concentrations are the lowest and which is particularly susceptible to ethanol-induced damage (French et al., 1993a). Estimates derived from in vitro studies indicate that the concentration of CYP2E1 in perivenous hepatocytes is sufficient to contribute significantly to hepatic oxygen consumption (Thurman et al., 1984a; 1984b; Tindberg and Ingelman-Sundberg, 1989). Consequently, the induction of CYP2E1 oxidase activity by repeated ingestion of ethanol and the resulting increase in microsomal oxygen consumption could further enhance the hepatic oxygen gradient resulting in hypoxic injury to pericentral hepatocytes.

1.2.7 NADPH-Cytochrome P450 Reductase

NADPH-cytochrome P450 reductase (EC 1.6.2.4: CYP-reductase) is a 78 kD flavoprotein that serves to transfer electrons from NADPH to cytochrome P450 and is an obligatory and often rate-limiting component in P450-dependent reactions (Kaminsky and Guengerich, 1985; Miwa et al., 1978). The enzyme is expressed in liver smooth endoplasmic reticulum (Phillips and Langdon, 1962) and nuclear membrane (Kasper, 1971), heart, lung, brain (Simmons and Kasper, 1989) and in neutrophils (Sakane et al., 1987; Laporte et al., 1991). The amino acid sequence of CYP-reductase has been determined in several mammalian species.
including humans (Haniu et al., 1989; Yamano et al., 1989), rats (Porter and Kasper, 1985), rabbits (Katagiri et al., 1986) and pigs (Haniu et al., 1986). The amino acid sequences are approximately 90% homologous between species. Unlike the cytochrome P450 family of genes, CYP-reductase appears to be encoded in humans by a single gene located on chromosome 7 (Yamano et al., 1989). The gene for the rat reductase has been cloned and shown to be composed of 16 exons (Porter et al., 1990).

*CYP-reductase* gene expression is regulated independently of P450 gene expression. The levels of CYP-reductase are generally lower than that of the cytochrome P450 enzymes and in most tissues the ratio is approximately 1:10 or less (Reed et al., 1986). CYP-reductase is induced by some cytochrome P450 inducers, however the magnitude of induction is generally smaller (Shen and Kasper, 1993). CYP-reductase expression varies at different stages of development. Liver CYP-reductase content is low during gestation (14% of adult levels) but increases three-fold at birth and reaches a peak at 2-3 months post-gestation (Shen and Kasper, 1993). After 35 days, mRNA levels decline while protein levels remain elevated, suggesting a role for posttranslational factors in the maintenance of CYP-reductase protein levels (Simmons and Kasper, 1989). As reported for CYP2E1, CYP-reductase is heterogeneously distributed within the liver lobule, with substantially higher levels of the enzyme expressed in perivenular hepatocytes (Kanai et al., 1992).

CYP-reductase is rate-limiting in the electron transfer reactions catalyzed by CYP2E1, and therefore plays an important indirect role in oxygen utilization and ROS formation by CYP2E1. Furthermore, studies using reconstituted membrane systems have shown that in the presence of iron and NADPH, CYP-reductase can undergo autoxidation, resulting in the reduction of oxygen to $O_2^-$ and the subsequent formation of $H_2O_2$ and $OH^-$ independently of CYP2E1 (Cederbaum, 1989; Winston and Cederbaum, 1983a). CYP-reductase may also participate in the formation of
HER, as suggested by the observation that anti-CYP2E1 IgG only inhibits HER formation in liver microsomes by roughly 50% (Albano et al., 1990).

Theoretically, increases in oxygen consumption and the formation of reactive oxygen species by liver microsomes following chronic ethanol treatment may be related to the level and activity of CYP-reductase, in addition to that of CYP2E1. The effect of chronic ethanol consumption on CYP-reductase is unclear. While increases in microsomal CYP-reductase activity have been reported in rats receiving chronic ethanol (Ekstrom and Ingelman-Sundberg, 1989; Joly et al., 1973; Lieber and DeCarli, 1970a; Shaw, 1989), other studies have failed to confirm this finding (French et al., 1993a; Badger et al., 1993a).

1.2.8 Neutrophils

A defining feature of many inflammatory diseases, including alcoholic hepatitis, is the presence of neutrophil infiltrates in the liver. Neutrophil-derived oxidants and proteolytic enzymes have been proposed to play an important role in mediating both the microbicidal activity of neutrophils and the cell injury associated with inflammation. The following section reviews the general mechanisms by which neutrophils can cause injury to host tissues and the evidence suggesting a role for neutrophils in ethanol-induced liver injury.

Neutrophils, also known as polymorphonuclear leukocytes (PMN) because of their multilobed nucleus, are formed in the bone marrow following the serial differentiation of pluripotent stem cells to committed progenitor cells of the neutrophil-granulocyte lineage, which ultimately give rise to functionally mature neutrophils. The maturation and differentiation of neutrophil progenitor cells in the bone marrow is controlled by the endogenous glycoproteins granulocyte-macrophage colony-stimulating factor (GM-CSF) (Cannistra and Griffin, 1988) and granulocyte colony-stimulating factor (G-CSF) (Clark and Kamen, 1987). In a normal human
adult there are roughly $2.5 \times 10^{10}$ neutrophils in the peripheral blood and an additional reserve of $2.5 \times 10^{12}$ neutrophils and their immediate precursors in the bone marrow (Van Oss, 1986). The production of neutrophils in the bone marrow normally occurs at a rate of approximately $10^{11}$ cells/day, however under some conditions, such as during periods of stress or infection, the rate of neutrophil differentiation can increase as much as 10-fold (Cannistra and Griffin, 1988). Once released into the circulation mature neutrophils have a relatively short life span of about one to two days (Smith, 1994).

Neutrophils constitute the body's first line of immunological defense against invading microorganisms (Schleimer et al., 1989). During the inflammatory response, chemotactic factors generated by infectious agents themselves, as well as those released as a result of their initial contact with phagocytes and other components of the immune system, signal the recruitment of neutrophils to sites of infection or injury. Under the influence of chemoattractants generated from the infection site, neutrophils in the blood stream penetrate the endothelial cell layer and migrate through connective tissue where they adhere to extracellular matrix components such as laminin and fibronectin (Cronstein and Weissmann, 1993; Nathan and Sanchez, 1990). The penetration of the endothelial layer by neutrophils, a process known as diapedesis, occurs at a rate of roughly 0.3 - 0.7 μm/sec (Bessis, 1973).

Neutrophils can effectively destroy a variety of non-self targets including bacteria, fungi, protozoa, viruses, virally infected cells and tumor cells (Ratcliffe et al., 1988). Ingestion of microorganisms begins with invagination of the neutrophil outer cell membrane which then proceeds to enclose the particle completely in a phagocytic vacuole or phagosome. Two microbicidal processes are activated concomitantly with phagocytosis: (i) the oxidative burst, so called because of the 50- to 100-fold increase in oxygen consumption, which results in the production of reactive oxygen species; and (ii) internalization of the phagosome and fusion with
neutrophil azurophillic and specific granules to form a phagolysosome. Exposure of the ingested particle within the microenvironment of the phagolysosome, to various reactive oxygen species and proteolytic enzymes, results in its destruction and digestion. Normally the lytic process does not affect surrounding host tissues because all the phagocytic reactions take place within the phagosome. However under some circumstances the contents may be released extracellularly, such as occurs when neutrophil targets are too large to be phagocytosed (frustrated phagocytosis) and when circulating neutrophils are activated prematurely (Klebanoff, 1992; Weiss, 1989).

1.2.8.1 Neutrophil-Derived Oxidants: Neutrophils have the intrinsic capacity to generate a variety of reactive oxygen species which contribute to both antimicrobial activity as well as the potentially destructive action on host tissues. The production of reactive oxygen species in neutrophils is regulated mainly by two enzymes, NADPH oxidase and myeloperoxidase.

*Superoxide and Hydrogen Peroxide:* NADPH oxidase (NADPH-FMN oxidoreductase: E.C. 1.6.99.1), is a membrane bound enzyme that catalyzes the transfer of electrons from NADPH to oxygen resulting in the formation superoxide:

\[ 2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + H^+ \]

Hydrogen peroxide is generated from the dismutation of superoxide, which occurs both spontaneously and by the action of the enzyme superoxide dismutase:

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

The electron transfer reaction catalyzed by NADPH oxidase also requires cytochrome b_{558} (Babior, 1992) and a flavin adenine dinucleotide (Babior and Kipnes, 1977), possibly NADPH-cytochrome P450 reductase (Sakane et al., 1987; Laporte et al., 1991). The importance of
NADPH oxidase activity in preventing infection of host tissues is clearly evident in individuals with chronic granulomatous disease (CGD). An X-linked form of CGD is caused by a gene defect (Royer-Pokara et al., 1986) that results in deficiency of cytochrome b558 and reduced NADPH oxidase activity (Segal et al., 1983; Tauber et al., 1983). Patients with CGD have high rates of infection by Staphylococcus aureus, Salmonella, Aspergillus and Candida (Tauber et al., 1983) and shortened life expectancy (Curnutte, 1993).

*Hypochlorous Acid (HOCl):* Myeloperoxidase (MPO: EC 1.11.1.7) is a heme-containing enzyme present in the primary (azurophilic) granules of neutrophils which, in combination with H₂O₂, can oxidize halides (i.e., Cl⁻, Br⁻, I⁻) and pseudohalides and (i.e., SCN⁻) to their corresponding hypohalous acids:

\[ \text{H}_2\text{O}_2 + \text{X}^- + \text{H}^+ \rightarrow \text{HOX} + \text{H}_2\text{O} \quad \text{(where X}^- = \text{Cl}^-, \text{Br}^-, \text{I}^- \text{or SCN}^-) \]

The resultant hypohalous acids, primarily HOCl, are powerful oxidizing agents that can react with numerous biological molecules producing changes similar to those that free radicals might generate. Neutrophils contain large amounts of MPO (up to 5% of the dry cell weight) which triggered cells can release in substantial amounts into the extracellular fluids (Klebanoff, 1992). Quantitative studies have estimated the rate of HOCl formation in maximally triggered neutrophils at roughly \(1 \times 10^{-7} \text{ mol/hour/}10^6 \text{ cells}\) (Test and Weiss, 1986). In addition, the cationic nature of MPO allows it to attach to cell surfaces, which further increases the local concentration of HOCl at the target cell surface (Selveraj et al., 1978).

Because of the high reactivity of HOCl, it does not actually accumulate in biologic systems but instead almost instantaneously disappears in multiple reactions with available substrates (Test and Weiss, 1986). HOCl participates in the formation of a derivative group of oxidants known as the chloramines. In this rapid and spontaneous reaction HOCl reacts with primary or secondary
amines that either are released from the neutrophil or are present in body fluids, thus generating a complex family of nitrogen-chlorine derivatives (Weiss et al., 1983). Depending on the nature of the R-group of the amine, the N-Cl oxidants may be either short-lived or long lived (Test et al., 1984). Thus, in contrast with HOCl which is highly reactive and therefore reacts primarily with membrane-associated targets, the less reactive chloramines can more readily diffuse across the plasma membrane to attack cytosolic components.

Hereditary deficiency of MPO is a common disorder, occurring at a frequency of 1:2000 to 1:4000 (Parry et al., 1981). Although MPO deficiency does not have as severe consequences as CGD (Parry et al., 1981), a predisposition to severe Candida infections (Klebanoff, 1992) and an increased incidence of neoplasms (Lanza et al., 1987) have been reported.

Reactive Nitrogen Species: In addition to reactive oxygen species, neutrophils can also form the nitrogen-centered free radical nitric oxide (NO•). Nitric oxide is formed during the conversion of L-arginine to L-citruline in a reaction catalyzed by the enzyme nitric oxide synthase (Moncada and Higgs, 1993). Nitric oxide causes relaxation of vascular smooth muscle and may act to facilitate the migration of neutrophils from blood vessels to surrounding tissues by causing vasodilation (Smith and Weidemann, 1993).

Hydroxyl Radicals: The involvement of OH• in neutrophil-mediated host tissue injury has been suggested from the observation that hydroxyl radical scavengers such as thiourea, dimethylsulfoxide and mannitol can protect target tissues from neutrophil-mediated damage in vitro and in vivo (Ward et al., 1983; Fox, 1984; Vercelotti et al., 1985; Weitberg et al., 1985). Neutrophils have the potential to generate OH•, however the biochemical pathways leading to
OH$^\cdot$ formation and its role in microbial killing and host tissue injury remains somewhat controversial.

NADPH oxidase may contribute to the formation of OH$^\cdot$ in neutrophils by generating O$_2^\cdot$ which dismutates to H$_2$O$_2$, leading to the formation of OH$^\cdot$ by the iron-dependent Haber-Weiss reaction (see 1.2.1.1). The biological significance of this pathway has been questioned on the basis that the extracellular concentration of iron is normally too low to promote the Haber-Weiss reaction, due to the presence of iron-binding proteins such as ferritin and lactoferrin (Cohen et al., 1988; Gutteridge et al., 1981). However under some circumstances, the liberation of iron from carrier proteins or iron release from damaged cells may occur resulting in the presence of catalytically active iron within the neutrophil microenvironment (Gannon et al., 1987). The observations that O$_2^\cdot$ can reductively mobilize iron from ferritin (Samokyszyn et al., 1988; Biemond et al., 1984) and that hydrogen peroxide can release iron from hemoglobin, which may infiltrate an injured tissue (Gutteridge, 1986), suggests that neutrophils may produce OH$^\cdot$ in vivo by the Haber-Weiss reaction.

An alternative mechanism for the formation of OH$^\cdot$ by neutrophils, that is MPO-dependent and iron-independent, has been proposed by Ramos et al. (1993) on the basis of in vitro studies using spin trapping and electron spin resonance. In the proposed reaction, HOCl generated by MPO reacts with O$_2^\cdot$, produced by NADPH oxidase, to form OH$^\cdot$

$$\text{HOCl} + \text{O}_2^\cdot \rightarrow \text{OH}^\cdot + \text{O}_2 + \text{Cl}^-$$

The relative contribution of the iron-dependent and iron-independent pathways to the formation of OH$^\cdot$ by neutrophils and the importance of these reactions in vivo remains to be determined.

A variety of cell types including neutrophils, which simultaneously generate NO$^\cdot$ and O$_2^\cdot$ (Rodenas et al., 1995; 1996), may also produce OH$^\cdot$ from the decomposition of peroxynitrite
Peroxynitrite is formed rapidly by the reaction of NO$^+$ and O$_2^-$, and decomposes to produce OH$^+$ by a mechanism that is iron-independent (Beckman et al., 1990):

\[
\text{NO}^+ + \text{O}_2^- \rightarrow \text{ONOO}^-
\]

\[
\text{ONOO}^- + \text{H}^+ \rightarrow \text{ONO}OH
\]

\[
\text{ONO}OH \rightarrow \text{OH}^+ + \text{NO}_2^-
\]

Very little is currently known regarding the relevance of this mechanism in vivo.

1.2.8.2 Proteolytic Enzymes and Serum α-1-Proteinase Inhibitor:

Neutrophil granules contain a large family of over 20 proteolytic enzymes, of which the serine proteases (e.g. elastase and cathepsin G) may have the greatest potential to act as mediators of tissue destruction in immunologic injury. Proteases (enzymes that cleave peptide bonds in the body of the protein chain) are able to attack key components of the extracellular matrix, which serve to maintain tissue architecture and play an important role in the repair of damaged tissue, and can mediate the dissolution of the extracellular matrix in vivo (Weiss, 1989).

Plasma and interstitial fluids contain a series of powerful antiproteinasases that regulate neutrophil elastase activity and prevent the enzyme from attacking extracellular substrates. Among these is α1-proteinase inhibitor (A1PI), a 52 kD glycoprotein that irreversibly inhibits neutrophil elastase by forming an enzyme-inhibitor complex. In vivo the calculated half-life of active elastase is only about 0.6 msec due to its rapid inactivation by A1PI. However in the absence of A1PI the half-life of elastase is increased to approximately 1200 msec (Travis and Salvesen., 1983; Janoff, 1985). There is considerable evidence to suggest that neutrophils are capable of circumventing the antiproteinase shield and are able to use their discharged elastase to attack and destroy host tissue. Human A1PI contains a critical methionine residue at position 358 of the polypeptide chain that is extremely sensitive to oxidation by triggered neutrophils.
Oxidation of Met_{358} causes a 2000-fold decrease in the rate of association between neutrophil elastase and the modified antiproteinase (Travis and Salvesen., 1983; Janoff, 1985). Although A1PI is present in extracellular fluids at relatively high concentrations, sufficient quantities of HOCl may be generated within the microenvironment of the neutrophil to cause oxidation of A1PI and solubilization of the connective tissue matrix (Weiss and Regiani, 1984; Weiss et al., 1986). Uninhibited neutrophil elastase, MPO and dysfunctional oxidatively-modified A1PI can be detected in the fluids recovered from areas of inflammation in several diseases including idiopathic pulmonary fibrosis (Cantin et al., 1987), rheumatoid arthritis (Velvart and Fehr, 1987; Edwards et al., 1988), emphysema (Damiano et al., 1986) and adult respiratory distress syndrome (Cochrane et al., 1983).

1.2.8.3 Role of Neutrophils in Alcoholic Liver Disease: There is a growing body of evidence to suggest that neutrophils may play a major role in the development of ALD. It is well recognized that neutrophil infiltration of the liver is a characteristic feature of the inflammatory reaction in alcoholic hepatitis (Takahashi et al., 1987; International Group, 1987) and that, an increase in the number of circulating neutrophils (neutrophil leukocytosis) is a common feature in ALD patients (Crapper et al., 1983; Hill et al., 1993; Khortus et al., 1991; Lischner et al., 1971). In addition, the degree of neutrophil infiltration in the liver is significantly correlated with mortality in ALD patients (French et al., 1993b).

The mechanisms responsible for the increase in neutrophils in the circulation and liver during ALD are not fully understood, but several factors involved in regulating the mobilization, adhesion and activation of neutrophils have been found to be altered by chronic ethanol consumption. Chronic ethanol intake is associated with elevated levels of endotoxin in the circulation and enhanced ability of endotoxin to trigger neutrophil adhesion in the hepatic
sinusoids (Ohki et al., 1996). Endotoxin is a heat stable toxin composed of complex lipopolysaccharide molecules and is a component of the outer membranes of many gram negative bacteria, such as those residing in the gastrointestinal tract. The levels of endotoxin in the systemic circulation are normally very low, but increased levels are observed in association with acute or chronic ethanol consumption and with ALD (Bode et al. 1987; Fukui et al., 1991, Goto et al., 1994; Schaeffer et al., 1997). The elevation may be caused by both: a decrease in the rate of endotoxin clearance from the systemic circulation (Fukui et al., 1993; McCuskey, et al. 1995, Fukui et al., 1994), a process mediated primarily by hepatic Kupffer cells; or by increased translocation of endotoxin from the gastrointestinal tract (Dobbs et al., 1994). Endotoxin stimulates hepatic Kupffer cells to release cytokines which in turn trigger neutrophil chemotaxis and the attachment and activation of neutrophils in inflamed tissues. The cytokines IL-1, IL-6, IL-8 and TNF-α are significantly elevated in the blood of patients with alcoholic hepatitis (Sheron et al., 1993; Hill et al., 1993, Khortus et al., 1991) and correlate with the clinical severity and mortality of acute alcoholic hepatitis (Bird et al., 1990; Sheron et al., 1991). In rat models of ethanol hepatotoxicity, administration of endotoxin potentiates liver injury (Bhagwandeen, 1987), while procedures that reduce circulating endotoxin levels, such as lactobacillus feeding or gut sterilization with the antibiotics polymyxin B or neomycin (Adachi et al., 1995), significantly reduces ethanol-induced liver damage.

The recruitment and activation of neutrophils can also be triggered by chemotactic factors produced during ethanol metabolism. Hepatocytes metabolizing ethanol generate metabolic byproducts that stimulate neutrophil chemotaxis (Shiratori et al., 1992a; 1992b; Roll et al., 1989). Activated complement produced by membrane bound antibody or antigen-antibody complexes also have the potential to attract neutrophils (Roit et al., 1989). Antibodies to acetaldehyde- and HER-modified proteins produced during the metabolism of ethanol in vivo
(Clot et al., 1995; 1996; Niemela et al., 1987; Koskinas et al., 1992) may therefore also play a role in the recruitment and/or activation of neutrophils in the liver during the progression of ALD.

Neutrophils present in the liver during ALD may contribute to tissue injury in several ways. First, reactive oxygen species generated by neutrophils can directly cause tissue injury. Secondly, neutrophil-derived HOCl and its secondary amine byproducts can inactivate circulating A1PI resulting in enhanced susceptibility of host tissue to degradation by proteolytic enzymes. Lastly, the physical presence of neutrophils in the liver sinusoids may impair hepatic blood flow and oxygen delivery.

The possible involvement of neutrophil-mediated inactivation of A1PI as a mechanism in ALD has not yet been extensively investigated, but is suggested by the enhanced susceptibility to spontaneous and alcohol-induced liver disease in individuals with genetic A1PI deficiency (Pott et al., 1983). The incidence of the heterozygous A1PI variant in patients with alcoholic liver disease is reported to be 250% higher than in the healthy population, while homozygotes for the mutant A1PI allele frequently develop spontaneous liver fibrosis and cirrhosis. Acetaldehyde, generated during ethanol metabolism, can also inactivate A1PI and may promote neutrophil-mediated proteolytic injury of the liver (Brecher et al., 1994; Brecher and Pavlock, 1992).

In addition to the deleterious effects of proteolytic enzymes and reactive oxygen species, neutrophils may also contribute to circulatory abnormalities associated with ALD. For example ischemic conditions, similar to that existing in the livers of patients with alcoholic cirrhosis and associated portal hypertension, may be sustained by plugging of capillaries with aggregates of activated neutrophils (McCord, 1987; Ricevetti et al., 1991). Bouts of acute AH, where massive infiltration of neutrophils occurs, may possibly disrupt blood flow and oxygen delivery to hepatocytes and therefore contribute to hypoxia. Interestingly, hypertension per se may contribute to oxidative liver injury, as suggested by the observation that hypertensive patients have
circulating neutrophils that are more oxidatively active than those of their normotensive counterparts (Pontremoli et al., 1989). It has been suggested that this represents an adaptive response on the part of the neutrophil to generate increased amounts of the vasodilator nitric oxide, in order to counteract the hypertensive state.

1.3 Therapy of Alcoholic Liver Disease (ALD)

Because the pathogenic mechanisms mediating ethanol-induced liver injury are not entirely understood, it has been difficult to develop specific and effective treatments for ALD. The objectives of ALD therapy can be summarized as follows: (i) to lessen the clinical severity of acute alcoholic hepatitis; (ii) to prevent the progression of the disease to more severe forms; and (iii) to reduce mortality. At the present time most therapeutic interventions for ALD are supportive and aim to counteract secondary complications arising from the disease, rather than the underlying destructive processes. General supportive measures for treatment include nutritional support, correction of vitamin deficiencies, dietary adjustment and diuretics for ascites and hepatic encephalopathy and specific treatment of complications such as bleeding and infections (Ramond et al., 1993).

Several studies have demonstrated that the prognosis for acute and chronic ALD is dramatically improved by abstinence from ethanol, while continued consumption is associated with increased morbidity and mortality (Borowsky et al., 1981). Nonetheless, in many patients ALD can progress even when alcohol consumption is discontinued (Marbet et al., 1987; Pares et al., 1986), and thus more effective therapeutic strategies must be employed.

Existing pharmacological treatments for ALD include corticosteroids, insulin and glucagon therapy, anabolic steroids and colchicine. In the majority of cases, clinical trials with these agents have either: (i) failed to demonstrate a significant therapeutic benefit; (ii) suffered
from methodological weaknesses such as heterogeneous patient populations, high patient dropout rates and small sample sizes, which precluded a reliable assessment; or (iii) the effects have not been consistently reproducible between studies (Ramond et al., 1993; Bloor et al., 1994).

Orthotopic liver transplantation is now considered a viable option for patients with end-stage ALD. Both patient and graft survival rates are at least comparable with the results in non-alcoholic liver transplant recipients (Bird et al., 1990; Lucey et al., 1992). Transplant is indicated in patients with ascites resistant to conventional diuretic therapy, repeated episodes of variceal bleeding, recurrent encephalopathy or small asymptomatic hepatocellular carcinomas (Sherman and Williams, 1994). Transplantation is less advisable in patients with acute alcoholic hepatitis, cardiomyopathy or central nervous system damage. In view of the shortage of organs available for transplant, concern has been expressed that transplant outcome may be adversely affected by continued alcohol consumption. Selection of only those patients who have been abstinent for a minimum of 6 months prior to acceptance has therefore been recommended (Schenker, 1984; Neuberger, 1989). While some reports have suggested that that this inclusion criteria may carry a significant risk of mortality (Bloor et al., 1994; Sherman and Williams, 1994; Flavin et al., 1988), other studies indicate that lack of abstinence for 6 months prior to transplantation may be a major predictive factor for relapse to alcohol abuse post-operatively. As a consequence, the U.S. National Institute of Health Executive Committee has recently recommended that the 6 month period of abstinence should be maintained as an inclusion criteria for liver transplantation in ALD patients (Hoofnagle et al., 1997).

It is apparent from the preceding discussion that the therapeutic options currently available for the treatment of ALD are highly limited and that the development of more effective treatments should be an urgent priority. Propylthiouracil is one of the few drugs which has shown promise in the treatment of ALD, yet surprisingly has been largely overlooked as a treatment option. The
general pharmacology of PTU, its role in preventing liver injury and the possible therapeutic mechanisms in ALD are reviewed in the following section.

1.3.1 General Pharmacology of Propylthiouracil

Propylthiouracil (6-n-propyl-2-thiouracil: PTU; Propyl-Uracil®) is a thioureylene compound first introduced over fifty years ago (Astwood and VanderLaan, 1945). The structure of PTU is similar to that of its parent compound thiouracil, consisting of a six-membered heterocyclic ring containing a thiourea moiety with the substitution of a propyl group (Figure 1).

PTU and the structurally related thioureylene compound methimazole (1-methyl-2-mercaptoimidazole: Tapazole®), are potent antithyroid agents and have been widely used in the treatment of hyperthyroidism in Graves' disease.

PTU is relatively insoluble in aqueous solutions at neutral pH (octanol: water partition coefficient = 4.95) (Hicks and Day, 1992), and is slowly and erratically absorbed from the gastrointestinal tract (Melander et al., 1977). In the treatment of Graves' disease PTU is given at an initial dose of 300 mg/day which is increased as necessary, occasionally to doses as much as 600 to 1000 mg/day, in order to normalize thyroid hormone function (Cooper, 1984). The peak serum level after 300 mg of PTU of approximately 40 μM varies linearly and is achieved within 1-2 hours after drug ingestion (Cooper et al., 1982; Long et al., 1983). The serum half-life of PTU in normal persons is about 75 minutes, and is not significantly affected by hyperthyroidism. Serum PTU concentrations of about 3 μg/ml (18 μM) are required to reduce thyroid activity by half (Cooper et al., 1982). PTU in the circulation is 75% bound to serum proteins, particularly albumin (Cooper, 1984), and is eliminated mainly through metabolism via hepatic conjugation (Alexander et al., 1969).
Figure 1: Chemical Structures of the Antithyroid Drugs Thiourea, Propylthiouracil and Methimazole.
PTU has been shown to inhibit both thyroid hormone synthesis and the peripheral conversion of thyroxine (T₄) to 3,3',5-triiodothyronine (T₃) (Taurog, 1974). The antithyroid effect of PTU stems from its ability to inhibit the enzyme thyroid peroxidase (TPO; donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7). TPO is a membrane bound hemoprotein which plays an essential role in the synthesis of thyroid hormones. TPO catalyzes the incorporation of oxidized iodide into tyrosine residues in the thyroid hormone precursor molecule thyroglobulin, as well as the intramolecular coupling of iodinated tyrosines leading to the formation of T₄ and T₃ (DeGroot and Niepołomische, 1977; Nunez, 1980). In vitro studies indicate that at low concentrations (10 μM) PTU can reversibly inhibit TPO-catalyzed thyroid hormone synthesis by competing with the tyrosyl residues of thyroglobulin for oxidized iodine (Taurog and Dorris, 1989; Taurog et al., 1995). At higher concentrations (50 μM) PTU is metabolized by TPO to form reactive metabolites which cause irreversible inhibition of the enzyme (Taurog et al., 1989; 1995).

1.3.2 Hepatoprotective Effect of Propylthiouracil

In addition to exerting an antithyroid effect, PTU has been shown to reduce liver injury in a variety of experimental paradigms, including that induced by hypoxia-reoxygenation in ethanol-fed rats (Israel et al., 1975; Britton et al., 1979), acetaminophen (Yamada et al., 1981; Linscheer et al., 1980; Raheja et al., 1982), carbon tetrachloride (Orrego et al., 1976), thioacetamide (Oren et al., 1996) and galactosamine (Cooper et al., 1984).

Studies in animals and clinical trials in humans indicate that PTU can also minimize ethanol-induced liver injury. Israel et al. (1975a) reported that treatment with PTU for 3 days at a dose of 50 mg/kg/day protected chronic ethanol-fed rats from hypoxia-induced centrilobular necrosis. There are three published clinical trials of PTU in the treatment of human ALD. Two of
the studies examined the effect of short-term PTU therapy while one study investigated long-term treatment. In a placebo-controlled randomized study of 133 patients with alcoholic hepatitis, short term PTU therapy (300 mg/day for 6 weeks) resulted in improvements in the clinical and histological parameters of liver injury in those patients with milder forms of the disease (Orrego et al., 1979). The therapeutic effect of PTU was not seen in patients with more severe hepatitis and PTU did not significantly reduce the overall rate of mortality. A second short-term study examined the effect of PTU therapy in 67 patients with severe AH (Halle et al., 1982). No significant difference in mortality was observed between the patients treated with PTU (7 of 31) and those receiving placebo (7 of 36). The patients in this study were similar to the subgroup identified in the first short-term study who had severe alcoholic hepatitis and showed a lesser response to PTU therapy than those with milder hepatitis. It should be pointed out that because of the relatively small number of patients, the probability of a type II error (failure to detect an effect of therapy when an effect actually exists) was high in both studies. In a long-term study of PTU in 310 patients with a wide spectrum of liver damage over a follow up period of two years, Orrego et al. (1987) reported a 48% reduction in cumulative mortality in PTU-treated patients with AH (13% mortality rate) as compared to patients receiving placebo (25%). Mortality in the subgroup of patients presenting the most severe symptoms of ALD, was reduced by over 50% from 55% in the placebo group to 25% in the PTU treatment group. It was further shown that the mortality rate was drastically reduced by PTU treatment in the subgroup of patients who did not continue to abuse ethanol. In this group the mortality rate was 2.8% as compared to 25% in the placebo group.

Despite evidence indicating the effectiveness of PTU in treating ALD, particularly in the sub-groups mentioned, it is not a widely accepted therapy. Presumably the reluctance of clinicians to prescribe PTU for the treatment of ALD is due in part to confusion arising from the
interpretation of clinical trials, concerns about side effects, and a lack of evidence for a definitive mechanism of action.

Although many clinicians believe that the use of antithyroid drugs is associated with frequent side effects, problems are encountered in only 1-5% of patients - a rate similar to that associated with many other commonly used drugs (Cooper, 1994). The possible side effects include fever, rash, urticaria, arthalgia or frank arthritis, and all seem to occur more frequently with higher doses (Cooper, 1984). Neutropenia is frequently observed in Grave's disease patients treated with PTU, occurring in up to 12% of adults and 25% of children, however the condition is benign and transient and not associated with an increased incidence of infections (Wing and Aspen, 1952; Amrheim et al., 1970). Agranulocytosis, a potentially life-threatening reduction in circulating granulocytes (< 250/mm³), may occur in roughly 0.5% of Grave's disease patients during PTU therapy (Trotter, 1962; Rosove, 1977). Toxic hepatitis and a lupus-like syndrome have also been reported in some Grave's disease patients receiving PTU, but are relatively uncommon (Huang and Liaw, 1995).

A recent review by Orrego et al. (1994) indicates that hypothyroidism and the side effects associated with PTU treatment in Grave's disease patients do not occur during the therapy of ALD. The dose of PTU used in ALD therapy (300 mg/day) is considerably lower than that typically used in the treatment of Graves disease (300-1000 mg/day) (Cooper, 1984), and is sufficiently low that patients do not develop obvious clinical hypothyroidism. Furthermore, since the two-year mortality rate associated with hypothyroidism is negligible as compared to a 25% mortality rate in untreated alcoholic hepatitis patients, the risk:benefit ratio of inducing hypothyroidism to prevent mortality from ALD is overwhelmingly in favor of this treatment.
1.3.3 Putative Mechanisms of Propylthiouracil Hepatoprotection

The original rationale for the use of PTU in the therapy of ALD is based on similarities between hyperthyroidism and the hypermetabolic state induced in the liver by chronic ethanol feeding (Israel et al., 1975b) and on the finding that PTU counteracts the increase in liver oxygen consumption associated with the ethanol-induced hypermetabolic state in rats (Israel et al., 1979). An association between thyroid hormones and liver disease has been suggested on the basis of several observations. Firstly, centrilobular necrosis has been described in patients with Grave’s disease, prior to the advent of definitive therapy and early diagnosis (Beaver and Pemberton, 1933; Weller, 1933). Secondly, administration of thyroid hormones has been shown to potentiate many forms of liver injury, such as that induced by lindane (Videla et al., 1995), 1,1-dichloroethylene (Kanz et al., 1988), thioacetamide (Oren et al., 1996), volatile anesthetics such as halothane, isoflurane and enflurane (Berman et al., 1983; Uetrecht et al., 1983) and ischemia-reperfusion (Troncoso et al., 1997). Finally, surgical removal of the thyroid gland provides a similar degree of protection as PTU against hypoxia-induced liver injury in ethanol-fed rats (Israel et al., 1975b) and thioacetamide hepatotoxicity (Oren et al., 1996).

Several additional observations indicate, however, that the modulation of thyroid hormones cannot be the only mechanism contributing to PTU hepatoprotection. It has been shown that the ability of PTU to protect against several forms of toxin-induced liver injury occurs independently of thyroid hormone levels. For example, galactosamine-induced hepatotoxicity is prevented by PTU in both hypothyroid and euthyroid rats and at doses which do not produce hypothyroidism, but is not affected by 2-thiouracil and 2-thiobarbituric acid, which are both potent antithyroid agents (Cooper et al., 1984). Similarly, the ability of PTU to prevent acetaminophen-induced liver injury is observed even when rats are made hyperthyroid by administration of T3 (Raheja et al., 1982). In ethanol-treated rats, S-Methyl-5-n-PTU - a synthetic
analog of PTU that is as effective as PTU in decreasing \( T_4 \) deiodination to \( T_3 \), but is much less potent in inhibiting thyroidal iodide incorporation - did not have similar potency to PTU in protecting against hypoxia-induced liver injury (Britton et al., 1979), indicating that the inhibition of peripheral deiodination does not contribute to PTU hepatoprotection.

PTU may protect against ethanol-induced liver injury by preventing hypoxia, although the underlying biochemical mechanisms have not been identified. PTU acutely increases oxygen delivery to the liver by an increase in portal blood flow (Kawasaki et al., 1989) which results in an increase in sinusoidal oxygenation (Matsunaga et al., 1991). Several in vitro studies have demonstrated the ability of PTU to prevent the ethanol-induced increase in hepatic oxygen consumption (Israel et al., 1975a; 1975b; Yuki et al., 1982). Using a novel in vivo technique for studying hepatic hemodynamics in conscious unrestrained rats, Carmichael et al. (1993) recently demonstrated that pretreatment with PTU for 5 days (50 mg/kg p.o) completely eliminates the increase in hepatic oxygen consumption resulting from chronic ethanol administration. The combination of increased blood supply and decreased oxygen utilization observed in this study could represent important contributions to the hepatoprotective effect of PTU. However there is no evidence to suggest that a similar effect is responsible for the ability of PTU to protect against other forms of toxin-induced liver injury, and as such cannot be invoked as a universal mechanism to account for the hepatoprotective effect of PTU.

The protection by PTU pretreatment against carbon tetrachloride- and acetaminophen-induced liver damage has frequently been attributed to a PTU-mediated increase in hepatic GSH concentration (Orrego et al., 1979; Yamada et al., 1981). Conceivably, PTU treatment could result in increased hepatic GSH levels by scavenging free radicals or reactive metabolites, by direct inhibition of free radical formation, or by altering the hepatic synthesis or turnover of GSH. Repeated administration of PTU in rats has been shown to increase the rate of GSH synthesis in
the liver without altering the turnover rate (Raheja et al., 1984). However, other studies on acetaminophen hepatotoxicity in rats indicate that PTU affords significant protection even when hepatic GSH is depleted by prior treatment with diethylmaleate (Raheja et al., 1983). Thus, the increase in hepatic GSH content alone is unlikely to be a primary mechanism of hepatoprotection.

Studies by Hicks and Day (1992) using cell free systems indicate that PTU can act as a free radical scavenger. PTU was shown to be an effective scavenger of hydroxyl radicals generated by 60Co-irradiation in an aqueous solution, as determined by steady state competition kinetics with the chromophore p-nitrosodimethylaniline (rate constant = 8 x 10^9 M⁻¹sec⁻¹). PTU was also shown to scavenge superoxide generated by the xanthine/xanthine oxidase system, however the magnitude of the effect was small (≈ 10%) and only occurred at a PTU concentration of 200 μM, which would be in excess of that achieved in vivo during PTU therapy. The biological significance of the hydroxyl radical scavenging effect of PTU in vitro is questionable since in vivo hydroxyl radical reacts with most molecules, with rate constants of ≥ 10^9 M⁻¹sec⁻¹ (von Sonntag, 1988). Furthermore, the molar concentration of PTU achieved in vivo is probably far less than that of endogenous molecules capable of rapidly reacting with OH-, including albumin (rate constant > 10^10 M⁻¹sec⁻¹) and glucose (rate constant ≈ 10^9 M⁻¹sec⁻¹) which are present at millimolar concentrations in body fluids (Smith et al., 1992; Halliwell 1995).

Treatment with PTU has been shown to result in a significant decrease in the hepatic content of transition metals such as iron and copper in rats (Al-Khayat et al., 1982). Increases in liver iron content are not uncommon in human ALD (see 1.2.1.4) and given the importance of iron in catalyzing the formation of hydroxyl radicals, may contribute to free radical-mediated liver injury. PTU has also been shown to prevent the increase in liver iron content associated with chronic ethanol treatment in mice (Gonzalez-Reimers et al., 1989), but a clear association
between the effect of PTU on hepatic iron content and the protection against hepatic injury remains to be conclusively demonstrated.

In summary, it is evident from the preceding discussion that no mechanism yet proposed can clearly account for the general hepatoprotective effect of PTU. There is however considerable and compelling recent evidence, as reviewed in the following discussion and demonstrated experimentally in this thesis, implicating the interaction of PTU with both (a) NADPH-cytochrome P450 reductase and (b) neutrophil myeloperoxidase, as probable therapeutic mechanisms. A combination of these mechanisms would account for both thyroid hormone-dependent and -independent hepatoprotection by PTU reported previously.

1.3.4 Effect of Propylthiouracil on NADPH-Cytochrome P450 Reductase

Thyroid hormones have been shown to regulate the expression and protein levels of CYP-reductase in the liver. In rats, a reduction in thyroid hormone levels following hypophysectomy or treatment with the antithyroid drug methimazole for 16-24 days, results in a substantial reduction in the levels of hepatic microsomal CYP-reductase protein, enzyme activity and mRNA, which can be restored to physiological levels by administration of T₄ (Ram and Waxman, 1992; Waxman et al., 1989). Two mechanisms were initially proposed to account for the thyroid dependence of CYP-reductase. First, the observed decrease in CYP-reductase in the hypothyroid state could involve a decrease in CYP-reductase mRNA, leading to decreased synthesis of the corresponding protein. Alternatively, newly synthesized CYP-reductase apoprotein might be more rapidly degraded in hypothyroid rats. In euthyroid rats treated with T₃ a significant induction of both CYP-reductase mRNA and protein levels occurs. However, while the major increase in protein levels occurs within 1 hour of treatment, the levels of mRNA do not increase until 8-12 hours after T₃ treatment (Waxman et al., 1989). These results suggest that the thyroid hormone-
mediated increase in mRNA is not responsible for the increase in CYP-reductase protein levels, and that stabilization of CYP-reductase apoprotein or an increase in the translatability of the pre-existing mRNA pool are more likely mechanisms. Recent studies have identified a thyroid hormone-responsive element in the promoter region of the CYP-reductase gene (bases -564 to 536) that contributes to the increase in CYP-reductase mRNA observed following T3 administration (O'Leary et al., 1997). It is possible that antithyroid drugs modulate CYP-reductase by a similar mechanism to T3, however this has not yet been confirmed experimentally. PTU has also been shown to decrease CYP-reductase activity (Raheja et al., 1985), but its effect on the levels of CYP-reductase protein and mRNA have not been reported.

The ability of PTU to modulate CYP-reductase activity represents a mechanism that could account for the drug's hepatoprotective effect. Since CYP-reductase is an obligatory and often rate-limiting component in P450-dependent reactions (Kaminsky and Guengerich, 1985; Miwa et al., 1978), a reduction in its activity may offset the potentially harmful effects on the liver associated with the ethanol-mediated induction of CYP2E1 (see 1.2.6.1). These effects include increases in (i) hepatic oxygen consumption (ii) the metabolism of CYP2E1 substrates and formation of reactive byproducts and (iii) the generation of reactive oxygen species. The possibility that the hepatoprotective effect of PTU may be mediated by such a mechanism, is suggested by the observation that PTU protects against liver injury by acetaminophen (Orrego et al., 1987) and carbon tetrachloride (Yamada et al., 1981; Linscheer et al., 1980; Raheja et al., 1982), both of which are converted by CYP2E1 to hepatotoxic metabolites. The ability of PTU to inhibit CYP2E1-catalyzed reactions via CYP-reductase may also explain the observations that ethanol-induced increases in hepatic oxygen consumption and ethanol metabolism are minimized by PTU treatment.
The modulation of CYP-reductase by PTU may also have protective effects against ethanol-induced liver injury independently of CYP2E1. In the presence of iron, CYP-reductase contributes to the formation of reactive oxygen species, oxygen utilization and ethanol metabolism without the involvement of CYP2E1 (Cederbaum, 1989; Winston and Cederbaum, 1983a). Some investigators have reported increases in hepatic CYP-reductase activity (Ekstrom and Ingelman-Sundberg, 1989; Joly et al., 1973; Lieber and DeCarli, 1970a; Shaw, 1989) and increased levels of hepatic iron (Shaw, 1989; Williams et al., 1967; Zimmerman et al., 1961) as a result of chronic ethanol ingestion, which suggests that the potentially damaging processes directly associated with CYP-reductase may be particularly active in the alcoholic.

1.3.5 Effects of Propylthiouracil on Neutrophils

A common feature in the various forms of toxin-induced liver injury which PTU can protect against is the presence of neutrophils in damaged tissues. Neutrophils have been implicated as mediators of tissue damage during inflammation (see 1.2.8) and may represent the locus at which PTU acts to prevent tissue injury. Evidence presented in the following section examines the possibility that PTU hepatoprotection involves either (i) a reduction in the total number of neutrophils in the body or (ii) inhibition of neutrophil myeloperoxidase.

1.3.5.1 Effect of Propylthiouracil on Neutrophil Count: One of the most common side effects associated with the use of PTU in the treatment of hyperthyroidism is transient leukopenia (white blood cell count below 4000 per mm³), typically involving a reduction in the number of circulating neutrophils. The incidence of PTU-induced leukopenia in Grave’s disease patients has been estimated at 12% in adults and 25% in children (Wing and Aspen, 1952; Amrheim et al., 1970). The condition is not associated with increased susceptibility to infection.
and due to its transient nature, often remains undetected unless the white blood cell count is closely monitored (Cooper 1984). A dose-dependent reduction in peripheral leukocytes has also been reported in rats treated with PTU (Kariya et al., 1983; 1984). Daily intraperitoneal administration of a relatively high dose of PTU (255 mg/kg i.p.) for 1 to 2 weeks was shown to reduce circulating leukocyte counts by 16% and 25%, respectively. The effect was reversible within 2 weeks after discontinuation of drug treatment and was not observed in rats receiving a single acute dose of PTU.

A more serious but considerably rarer complication observed in hyperthyroid patients treated with PTU or methimazole is agranulocytosis. This condition is characterized by fever, systemic toxicity, bacterial infection and a granulocyte count below 250 per mm³ and typically close to zero. The incidence of PTU- and methimazole-induced agranulocytosis is approximately 0.5% and the risk appears to be highest during the first three months of therapy (Trotter, 1962; Rosove, 1977; Cooper et al., 1983). Recovery from this condition normally occurs within 7-14 days after discontinuation of treatment, however this can be accelerated by restoring circulating neutrophil counts through treatment with G-CSF (Balkin et al., 1993; Magner and Snyder, 1994).

Based on the potential of PTU to induce agranulocytosis it has been postulated that PTU or its metabolites may be directly toxic to peripheral neutrophils and/or bone marrow precursor cells, or may trigger an autoimmune response. PTU is oxidized by neutrophil myeloperoxidase resulting in the formation of reactive metabolites such as PTU-sulfenyl chloride and propyl-2-sulfonate that are capable of binding to sulfhydryl groups in proteins (Lam and Lindsay, 1988; Lee et al., 1988; Waldhauser and Utrecht, 1991). These modified proteins may be recognized as foreign by the immune system, resulting in the production of anti-neutrophil antibodies. Such antibodies have been detected in the serum of patients with PTU-induced agranulocytosis (Bilezikian et al., 1976; Guffy et al., 1984; Fibbe et al., 1986).
Agents which reduce the availability of neutrophils in the circulation (i.e., cyclophosphamide) or inhibit neutrophil migration (i.e., colchicine) have previously been shown to minimize alcohol- and other forms of toxin-induced tissue injury (Kershenobich et al., 1988; Kanazawa et al., 1992; Ghio et al., 1991). In theory, a reduction in circulating neutrophils would minimize their infiltration into injured tissues and the subsequent release of proteolytic enzymes and reactive oxygen species thought to contribute to tissue damage. The ability of PTU to reduce the number of available neutrophils in the body may therefore be a mechanism contributing to the drug's hepatoprotective effects. This possibility was investigated in the experiments described in section 4 (Study 2).

1.3.5.2 Effect of Propylthiouracil on Neutrophil Myeloperoxidase: Analysis of the amino acid and nucleotide sequences of myeloperoxidase (MPO) and thyroid peroxidase (TPO) reveals a striking degree of homology (Johnson et al., 1987; Morishita et al., 1987), indicating that MPO and TPO are members of the same gene family and diverged from a common ancestral gene (Libert et al., 1987, Kimura and Ikedo-Saito, 1988). As a consequence of these structural similarities, antithyroid drugs, which are potent inhibitors of TPO, are equally effective as inhibitors of MPO. MPO catalyzes the \textit{in vivo} formation of hypohalous acids such as HOCl by activated neutrophils (see 1.2.8) and also catalyzes the \textit{in vitro} iodination of thyroid hormone precursors and the intramolecular coupling of iodinated tyrosine residues to form T	extsubscript{3} and T	extsubscript{4}, in a manner similar to that described for TPO. Antithyroid drugs such as PTU and MMI have been shown to inhibit MPO-catalyzed iodination and coupling (Taurog and Doris, 1992), as well as the chlorinating activity of the enzyme (Lee et al., 1990), suggesting that MPO inhibition involves mechanisms similar to those previously described for the inhibition of TPO by antithyroid drugs (Taurog and Doris, 1992; Taurog and Doris, 1989). The mechanism of inhibition appears to
involve oxidation and subsequent conformational change of the heme group of MPO which enables PTU to bind to it and cause irreversible inhibition of the enzyme (Lee et al., 1988; Engler et al., 1982).

MPO is thought to contribute to several forms of inflammatory tissue injury by generating HOCl which can (a) inactivate A1PI thereby allowing proteolytic damage to occur and (b) generate the highly toxic hydroxyl radical (see 1.2.8 and 1.2.8.2). Direct inhibition of MPO activity and scavenging of HOCl generated by MPO are mechanisms which have been proposed to contribute to the anti-inflammatory properties of a variety of therapeutic agents, including non-steroidal anti-inflammatory drugs (Shacter et al., 1991; Dallegrí et al., 1992a; 1992b; 1990a; 1990b), aminoglycoside antibiotics (Cantin and Woods, 1993) and anti-ulcer medications (Van Zyl et al., 1993).

The ability of antithyroid drugs to inhibit MPO may be an important therapeutic mechanism in the prevention of tissue injury, since a reduction in either HOCl-mediated inactivation of A1PI or HOCl-mediated hydroxyl radical formation would be a likely result. The effect of antithyroid drugs on neutrophil-mediated inactivation of A1PI and on the ability of neutrophils to generate hydroxyl radicals and other reactive oxygen species was investigated in the studies described in section 5 (Study 3).
2. AIMS OF THE INVESTIGATION

The antithyroid drug PTU has been shown to markedly reduce liver injury and mortality associated with alcoholic liver disease and to protect against a variety of other forms of toxin-induced liver injury, although the underlying mechanisms have not been determined. The present investigations examined several possible mechanisms which could account for the hepatoprotective properties of PTU, including (a) inhibition of the microsomal enzyme cytochrome P450 reductase (CYP-reductase); (b) a reduction in circulating neutrophil counts and (c) inhibition of the neutrophil enzyme myeloperoxidase.

Chronic ethanol consumption is associated with induction of the cytochrome P450 isozyme CYP2E1. CYP2E1 is involved in the metabolism of ethanol and its induction can result in (i) elevated production of potentially damaging reactive oxygen species, such as the hydroxyl radical (ii) increased formation of acetaldehyde, a highly reactive oxidative byproduct of ethanol metabolism and (iii) enhanced consumption of oxygen leading to hypoxia. Prior studies have shown that reduced levels of circulating thyroid hormones are associated with a corresponding reduction in the levels of CYP-reductase, a rate-limiting enzyme in the reactions catalyzed by CYP2E1. The present studies examined whether this mechanism could account for the ability of PTU to minimize liver injury induced by ethanol and other hepatotoxins. Experiments were conducted to determine the effect of PTU on the levels and activity of CYP-reductase in liver microsomes and to determine whether PTU could prevent elevations in oxygen utilization, acetaldehyde formation and the generation of reactive oxygen species associated with CYP2E1 induction in chronic ethanol- and acetone-treated rats.

The infiltration of neutrophils in damaged tissues is a hallmark characteristic of liver injury induced by ethanol and many other hepatotoxins. Neutrophils are thought to promote tissue injury through the release of reactive oxygen species and proteolytic enzymes. Prior studies suggest that
PTU may cause a reduction in circulating neutrophils, an effect which in theory could prevent their ability to cause cell damage at sites of inflammation. Experiments presented in this thesis examined the relationship between circulating neutrophil counts and liver injury in rat models of alcoholic liver disease, and assessed the effect of PTU on circulating neutrophil counts in these animals.

Myeloperoxidase is a neutrophil enzyme that generates reactive oxygen species that can (i) be directly toxic to cells, (ii) generate hydroxyl radicals or (iii) cause inactivation of α-1-proteinase inhibitor (A1PI), an endogenous protein that normally protects tissues from injury by neutrophil-derived proteolytic enzymes. *In vitro* studies indicate that antithyroid drugs are potent inhibitors of myeloperoxidase, and may also act as hydroxyl radical scavengers - effects which may underlie the cytoprotective properties of these agents. Experiments were conducted to determine the effect of PTU on myeloperoxidase activity and in particular, the ability of neutrophils to generate hypochlorous acid and hydroxyl radicals, and to promote the oxidative inactivation of A1PI.
3. STUDY 1

EFFECT OF PROPYLTHIOURACIL TREATMENT ON NADPH-CYTOCHROME P450 REDUCTASE LEVELS, OXYGEN CONSUMPTION AND HYDROXYL RADICAL FORMATION IN LIVER MICROSOMES FROM RATS FED ETHANOL OR ACETONE CHRONICALLY.⁴

3.1 Rationale:

The cytochrome P450-dependent microsomal monooxygenase system is involved in the metabolism of a variety of xenobiotic agents. The hepatic P450 isozyme P4502E1 (CYP2E1) is a major component of the ethanol-inducible microsomal ethanol oxidizing system (MEOS) (Johansson et al. 1988; Lieber 1988; Ryan et al., 1986) and has been postulated to play a role in the pathogenesis of ethanol-induced liver injury (French et al. 1993; Ingelman-Sundberg, et al. 1993; Morimoto et al., 1993). Some of the proposed mechanisms by which CYP2E1 induction may contribute to ethanol-induced liver injury include oxidative stress due to increased formation of reactive oxygen species (Ekstrom and Ingelman-Sundberg, 1989) and hypoxic damage resulting from increased microsomal oxygen consumption. A possible role for CYP2E1 in promoting intracellular hypoxia is based on the observations that: (ii) CYP2E1 displays an exceptionally high degree of oxidase activity, and with NADPH as a cofactor, can reduce oxygen and generate reactive oxygen species in the absence of ethanol or other substrates (Ekstrom and Ingelman-Sundberg, 1989; Gorsky et al., 1984); and (ii) CYP2E1 is expressed and induced primarily in zone III of the liver acinus (Buhler et al., 1991; Ingelman-Sundberg et al., 1988), a region which is particularly susceptible to ethanol-induced damage (French et al., 1993).

Increases in oxygen consumption and the formation of reactive oxygen species by liver

⁴ Ross et al. (1993)
microsomes following chronic ethanol treatment may also result from an elevated content or activity of the flavoprotein NADPH-cytochrome P450 reductase (E.C.1.6.2.4: CYP-reductase) (Joly et al., 1973; Lieber and DeCarli, 1970b; Shaw, 1989), which serves to transfer electrons from NADPH and is an obligatory and often rate-limiting component in P450-dependent reactions (Kaminsky and Guengerich, 1985; Miwa et al., 1978). Studies using reconstituted membrane systems have shown that in the presence of iron and NADPH, CYP-reductase can undergo autoxidation, resulting in the reduction of oxygen to O$_2^\cdot$ and H$_2$O$_2$ independently of CYP2E1 (Cederbaum, 1989; Winston and Cederbaum, 1983a). Through Fenton-type and iron-catalyzed Haber-Weiss reactions, O$_2^\cdot$ and H$_2$O$_2$ can in turn form OH$^\cdot$ (Cederbaum, 1989; Winston and Cederbaum, 1983a), which is highly reactive and may promote liver injury through oxidative modification of membrane lipids (Cross et al., 1987; Kaplowitz et al., 1986), proteins (Bielski and Shiue, 1979) and nucleic acids (Cochrane et al., 1988).

The antithyroid drug propylthiouracil (PTU) has been shown to significantly minimize ethanol-induced liver injury in a rat model (Israel et al., 1975a) and to markedly reduce the risk of mortality from alcoholic liver disease in humans (Orrego et al., 1987). The efficacy of PTU in attenuating ethanol-induced liver injury may be explained by the observation that increases in hepatic oxygen utilization as a result of chronic ethanol ingestion in rats can be prevented by administration of PTU (Carmichael et al., 1993; Rachamin et al., 1985; Thurman et al., 1984a; Thurman et al., 1984b). The underlying biochemical mechanisms responsible for the reduction in oxygen consumption by PTU have not yet been determined. In rats, depletion of thyroid hormones by hypophysectomy (Ram and Waxman, 1992; Rumbaugh et al., 1978; Waxman et al., 1989) or more selectively by treatment with the antithyroid drugs methimazole or PTU (Ram and Waxman, 1992; Raheja et al., 1985) has been shown to lead to a substantial reduction in hepatic microsomal CYP-reductase activity and mRNA levels. Since CYP-reductase can contribute to
microsomal oxygen consumption and the production of reactive oxygen species, the hepatoprotective effect of PTU may be due to its ability to lower CYP-reductase levels and thereby minimize increases in microsomal oxygen consumption and the generation of reactive oxygen species associated with chronic ethanol ingestion. In the present study we have examined the effect of chronic ethanol and PTU administration on hepatic microsomal CYP-reductase, oxygen consumption, OH− formation and on CYP2E1 levels and the microsomal metabolism of the CYP2E1 substrates, ethanol and N,N-nitrosodimethylamine (NDMA). The effect of PTU was further investigated in rats receiving acetone chronically, a procedure which has also been shown to induce hepatic CYP2E1 (Hétu and Joly, 1988; Koop and Casazza, 1985; Sakai et al., 1992).

3.2 Method

3.2.1 Materials

Acetylacetone, cytochrome c, NADPH, N,N-nitrosodimethylamine (NDMA), semicarbazide HCl, sodium azide and Tris-HCl were obtained from the Sigma Chemical Co. (St. Louis, MO). Acetaldehyde, acetone, dimethylsulfoxide (DMSO), formaldehyde and sodium EDTA were purchased from BDH Ltd. (Poole, U.K.). Chelex-100 metal chelating resin (100-200 mesh) and SDS-PAGE reagents were from BioRad Laboratories (Richmond, CA). CYP2E1 and CYP-reductase antibodies were from Oxygene (Dallas, TX) and bisacrylamide was from Schwarz-Mann Biotech (Cleveland, OH). All other reagents were of the highest grade commercially available.

3.2.2 Chronic Ethanol Administration

Female Wistar rats (Charles River, Quebec) weighing 125-135 g were given continuous access for 29 days to a liquid diet containing ethanol or an isocaloric diet. Two additional groups
of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diet during the last 10 days of ethanol treatment\(^5\). Diets were delivered by an automated 4-way feeding system to ensure equal consumption between groups, using a modified version of a technique described by Israel et al. (1984). Liquid diets had a total caloric content of 1 kcal/ml with 32% of total calories supplied as fat (a mixture of 80% corn oil, 12% olive oil and 8% cod liver oil), 9% as carbohydrates (maltose dextrin), 23% as protein (casein) and 36% as ethanol or additional carbohydrates. Diets also contained 1.505 g/L vitamin mix #20315 (Bioserv, Frenchtown, NJ), 10.03 g/L Bioserv salt mix #711, 4 g/L viscarin, 269 mg/L DL-methionine and 228 mg/L choline chloride. The exact diet formulations are listed in Appendix 1.

3.2.3 Chronic Acetone Administration

Female Wistar rats (175-225 g) were treated for 14 days with either 1% (v/v) acetone in the drinking water or plain tap water. Two additional groups of acetone-treated and control rats concurrently received PTU (25 mg/kg/day) or vehicle (water) by intragastric infusion for 14 days. Rat chow was available ad libitum throughout the experiment.

3.2.4 Surgical Procedures and Isolation of Liver Microsomes

On the morning of surgery, diets were withheld and surgery was performed under ether anesthesia. After collecting unheparinized blood samples from the descending aorta, livers were perfused in situ with 30 ml of ice cold phosphate buffered saline, then removed and homogenized in cold 10 mM sodium/potassium phosphate buffer (pH=7.4) containing 1.14% KCl. Microsomes were separated by centrifugation of the homogenate at 10000 x g for 10

\(^5\) The feeding of ethanol-containing liquid diets by the method described above has been shown to produce morphological and histological signs of liver injury within 28 days of treatment (Lieber and DeCarli, 1970c).
minutes and ultracentrifugation of the supernatant at 100000 x g for 60 minutes. The microsomal pellet was washed once in buffer, resuspended in 2 ml of 50 mM potassium phosphate buffer and stored at -70 °C at a protein concentration of approximately 30 mg/ml. In the acetone study, all solutions for liver preparation and biochemical assays were prepared with distilled water treated with Chelex-100 to remove contaminating iron.

3.2.5 Analytical Procedures

3.2.5.1 Thyroid Hormones: The levels of thyroxine (T₄) and triiodothyronine (T₃) were determined in serum by ¹²⁵I radioimmunoassay (Hormone Research Laboratory, St. Michael’s Hospital, Toronto).

3.2.5.2 Protein Determination: Protein concentrations in microsomal suspensions were measured using BioRad protein assay dye reagent (Bradford, 1976) and bovine serum albumin standards.

3.2.5.3 Microsomal Oxygen Consumption: Oxygen consumption was measured using a Clarke oxygen electrode (YSI, Yellow Springs, OH) in incubation mixtures containing 1 mg microsomal protein in 50 mM potassium phosphate buffer (pH = 7.4), and 50 μM EDTA. After a 3 minute preincubation period, 1 mM NADPH was added to the reaction mixture and oxygen consumption was measured continuously for 3 minutes at 37 °C.

3.2.5.4 Total P450: Total P450 content in liver microsomal suspensions was determined by the method of Omura and Sato (1964; Estabrook and Werringloer, 1978).
3.2.5.5 **CYP-reductase Activity:** CYP-reductase activity was assayed at 30 °C in 1 ml incubation mixtures consisting of 300 mM potassium phosphate buffer (pH = 7.7), 70 nmol cytochrome c and 30 μg microsomal protein. Reactions were initiated by addition of 1 mM NADPH and the rate of cytochrome c reduction was determined spectrophotometrically at 550 nm based on an extinction coefficient of 21 mM⁻¹cm⁻¹ (Stroebel and Dignam, 1978).

3.2.5.6 **NDMA Oxidation:** The oxidation of N,N-nitrosodimethylamine (NDMA) to formaldehyde was determined as described previously (Tu *et al.*, 1981). Reaction mixtures contained 50 mM Tris-HCl buffer (pH = 7.4), 150 mM KCl, 10 mM MgCl₂, 1 mM NDMA and 1 mg microsomal protein. After a 2 minute preincubation period the reaction was initiated with 0.5 mM NADPH and incubated for 30 minutes at 37 °C. The reaction was terminated with 25% (w/v) ZnSO₄ and saturated Ba(OH)₂. The mixture was centrifuged and 700 μl of supernatant was combined with 300 μl of concentrated Nash reagent (5 g ammonium acetate and 70 μl acetylacetone in 6 ml of 3% acetic acid) (Nash, 1953). Following a 30 minute incubation at 50 °C the absorbance at 412 nm was measured and the formaldehyde concentration was determined by comparison with standards of known concentration.

3.2.5.7 **MEOS Activity:** Microsomal ethanol oxidation was measured according to the method of Lieber and DeCarli (1970a) in 25 ml Erlenmeyer flasks with center wells containing 15 mM semicarbazide HCl in 100 mM potassium phosphate buffer. The reaction mixture in the outer well consisted of 50 mM potassium phosphate buffer (pH = 7.4), 1 mM sodium azide (to inhibit catalase present as a contaminant in isolated microsomes), 50 mM ethanol, 5 mM MgCl₂, 1 mM sodium EDTA and 3 mg microsomal protein. After a 5 minute preincubation, 1 mM NADPH was added, the vessels were sealed and incubated at 37 °C for 5 minutes. The reaction was then
terminated with 70% (w/v) trichloroacetic acid. After standing overnight at room temperature, the formation of semicarbazide-acetaldehyde complex was determined by measuring absorbance at 224 nm as compared to standards of known concentration (Gupta and Robinson, 1966).

3.2.5.8 Microsomal OH⁻ Formation: The production of OH⁻ and OH⁻-like oxidizing species was assayed by measuring the generation of formaldehyde from DMSO (Cederbaum and Cohen, 1984). Microsomes (0.25 mg) were suspended in 100 mM potassium phosphate buffer (pH = 7.4), 1 mM sodium azide, 25 μM ferric ammonium sulfate/50 mM EDTA (ferric EDTA 1:2 chelate) and 50 mM DMSO. After a 2 minute preincubation at 37°C, the reaction was initiated with 1.5 mM NADPH, terminated after 10 minutes by addition of 4% (w/v) trichloroacetic acid and then centrifuged. To determine formaldehyde concentration, 700 μl of supernatant was combined with 300 μl of concentrated Nash reagent and incubated for 30 minutes at 50 °C (Nash, 1953). The absorbance at 412 nm was measured and the formaldehyde concentration was determined by comparison with standards of known concentration.

3.2.5.9 Western Blot Determination of CYP2E1 and CYP-reductase: Microsomal protein (6 μg) was resolved by electrophoresis in a 7.5% (w/v) SDS polyacrylamide gel and transferred electrophoretically to nitrocellulose. Immunoblotting was performed with rabbit:anti-rat cytochrome P4502E1 or rabbit:anti-rat NADPH-cytochrome P450 reductase (Oxygene, Dallas, TX), diluted 1:100, followed by alkaline phosphatase-linked goat-anti rabbit IgG (diluted 1:1000). The relative density of immunostained bands was quantified by computerized densitometry using a BioRad Imaging Densitometer (#GS-670) and BioRad Molecular Analyst software.
3.2.6 Statistical Analysis

All values presented represent means ± SEM of 6-8 rats per treatment group.

Significant differences between groups were determined by analysis of variance followed by post-hoc Duncan’s Test. Results were considered significant at p < 0.05.

3.3 Results

3.3.1 Chronic Ethanol/PTU Study

Rats receiving ethanol diets consumed an average of 13.2 ± 0.2 g ethanol/kg/day during the 29 day treatment period. The rate of body weight gain during the course of the experiment was similar between the groups and was not affected by either chronic ethanol treatment or by PTU administration (Table 2). Rats receiving PTU in the liquid diet received an average dose of 24.3 ± 0.6 mg PTU/kg/day. This treatment resulted in a 64% reduction in the levels of circulating T₃ and T₄ (Table 2). The effect of PTU on thyroid hormones was similar in both control and ethanol-treated rats, while ethanol treatment itself did not affect T₃ or T₄ levels.

In agreement with prior studies (Ram and Waxman, 1992; Rumbaugh et al., 1978; Waxman et al., 1989; Raheja et al., 1985), induction of hypothyroidism was associated with a decrease in liver microsomal CYP-reductase activity (Figure 2). Administration of PTU resulted in a 27% decrease in the rate of microsomal cytochrome c reduction in both ethanol-treated rats (ethanol diet = 216.2 ± 7.7 nmol/mg/min vs. ethanol diet + PTU = 158.1 ± 13.5 nmol/mg/min) and in controls (control diet = 186.0 ± 14.6 nmol/mg/min vs. control diet + PTU = 137.2 ± 15.0 nmol/mg/min). CYP-reductase activity in rats treated with ethanol or ethanol + PTU was increased by 15% as compared to the corresponding control groups, however the effect was not statistically significant (p = 0.06). PTU treatment was also associated with a reduction of CYP-reductase protein levels as determined by Western blots. Figure 3 shows a decrease in immunoreactivity of a single 78 kD
Table 2: Effects of chronic ethanol and PTU treatments on body weight and serum levels of thyroid hormones.*

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Total T&lt;sub&gt;4&lt;/sub&gt; (nmol/L)</th>
<th>Total T&lt;sub&gt;3&lt;/sub&gt; (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>180 ± 3</td>
<td>74.1 ± 2.7</td>
<td>1.24 ± 0.07</td>
</tr>
<tr>
<td>Ethanol diet</td>
<td>179 ± 4</td>
<td>78.5 ± 5.0</td>
<td>1.47 ± 0.07</td>
</tr>
<tr>
<td>Control diet + PTU</td>
<td>180 ± 3</td>
<td>25.0 ± 4.1†‡</td>
<td>0.40 ± 0.04†‡</td>
</tr>
<tr>
<td>Ethanol diet + PTU</td>
<td>183 ± 5</td>
<td>28.9 ± 4.7†‡</td>
<td>0.60 ± 0.12†‡</td>
</tr>
</tbody>
</table>

* Rats were fed isocaloric amounts of either ethanol-containing or control liquid diets for 29 days. Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diets for 10 days. Values are means ± SEM (n = 7 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by post-hoc Duncan's Test.
† p < 0.05, compared with the control group.
‡ p < 0.05, compared with the ethanol-treated group.
Figure 2: Effect of PTU on NADPH-cytochrome P450 reductase (CYP-reductase) activity in liver microsomes from ethanol-treated rats. PTU (average dose = 24.3 ± 0.6 g/kg/day) was administered for 10 days to rats receiving either ethanol-diet or control diet for 29 days. CYP-reductase activity was determined by the rate of cytochrome c reduction at 550 nm. Vertical bars indicate mean ± SEM (n=7 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Figure 3: Immunoblot analysis of NADPH-cytochrome P450 reductase (CYP-reductase) content in liver microsomes isolated from rats receiving (A) control diet (B) ethanol diet (C) control diet + PTU or (D) ethanol diet + PTU (n=4 per group). The left hand lane contained pre-stained protein standards with molecular weights as indicated. 6 μg of microsomal protein was separated electrophoretically in 7.5% SDS-polyacrylamide gels and immunoblotted with rabbit anti-rat reductase IgG. The rabbit anti-rat CYP-reductase IgG recognized a single 78 kD protein band corresponding to CYP-reductase.
protein band recognized by rabbit anti-rat CYP-reductase IgG in microsomes from PTU-treated rats. In microsomes from control rats treated with PTU, the density of the CYP-reductase immunoreactive band was decreased by 69 ± 15% (n=4) as compared to untreated rats. In ethanol-fed rats receiving PTU, CYP-reductase immunoblot density was decreased by 66 ± 11% (n=4) as compared to microsomes from rats receiving ethanol alone. Overall the effect of PTU was significant only in ethanol-treated animals (p = 0.05). CYP-reductase immunoreactivity in microsomes from ethanol- and ethanol + PTU-treated rats showed a 46 ± 23% and a 53 ± 47% increase, respectively, as compared to their corresponding control groups, however these effects were not statistically significant.

The rate of hepatic microsomal oxygen consumption (Figure 4) in rats receiving ethanol for 29 days (19.4 ± 1.8 nmol/mg/min) was 70% higher as compared to rats receiving isocaloric control diets (11.4 ± 0.9 nmol/mg/min). The ethanol-induced increase in oxygen consumption was partially prevented by PTU treatment. Coadministration of PTU reduced the rate of oxygen consumption by 25% in rats receiving either ethanol-diet (14.5 ± 1.4 nmol/min/mg) or control diet (8.6 ± 1.3 nmol/min/mg).

The production of OH· and OH·-like oxidizing species, as determined by the rate of formaldehyde production from DMSO, was elevated by 49% in microsomes from ethanol-treated rats (Figure 5). PTU treatment significantly reduced the rate of hydroxyl radical formation in microsomes from ethanol-treated rats (25%).

The effect of chronic ethanol and PTU administration on microsomal P450 content and the metabolism of NDMA and ethanol are summarized in Table 3. Microsomes from ethanol-treated rats showed an increase in total microsomal cytochrome P450 content (48%), and in the rate of ethanol oxidation (50%) and a 2-fold increase in the rate of demethylation of NDMA to formaldehyde. PTU treatment also produced a significant increase in P450 content in microsomes.
Figure 4: Effect of chronic ethanol administration and PTU treatments on the rate of NADPH-dependent oxygen consumption in rat liver microsomes. Rats were fed ethanol diet or control diet for 29 days. PTU was administered concurrently during the last 10 days of the experiment. Vertical bars indicate mean ± SEM (n=7 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Figure 5: Effect of chronic ethanol and PTU treatments on the rate of microsomal production of hydroxyl radicals as determined by the formation of formaldehyde from DMSO in the presence of 1.5 mM NADPH and 25 mM:50 mM ferric:EDTA. Vertical bars indicate mean ± SEM (n=7 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Table 3: Effects of ethanol and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA.*

<table>
<thead>
<tr>
<th></th>
<th>Total P450 (nmol/mg)</th>
<th>NDMA (nmol/mg/min)</th>
<th>MEOS (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control diet</td>
<td>1.00 ± 0.06</td>
<td>1.08 ± 0.04</td>
<td>8.3 ± 0.9</td>
</tr>
<tr>
<td>Ethanol diet</td>
<td>1.49 ± 0.09†</td>
<td>2.17 ± 0.09†</td>
<td>12.5 ± 1.0†</td>
</tr>
<tr>
<td>Control diet + PTU</td>
<td>1.29 ± 0.09†</td>
<td>1.00 ± 0.03‡</td>
<td>6.6 ± 0.6‡</td>
</tr>
<tr>
<td>Ethanol diet + PTU</td>
<td>1.57 ± 0.05†‡$</td>
<td>2.16 ± 0.08†‡$</td>
<td>9.7 ± 1.0‡$</td>
</tr>
</tbody>
</table>

* Rats were fed isocaloric amounts of either ethanol-containing or control liquid diets for 29 days. Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diets for 10 days. Values are means ± SEM (n = 7 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by post-hoc Duncan's Test.
† p < 0.05, compared with the control group.
‡ p < 0.05, compared with the ethanol-treated group.
§ p < 0.05, compared with the PTU-treated control group.
from control rats but not from ethanol-treated rats. While the rate of NDMA metabolism was not altered in microsomes from PTU-treated rats, MEOS activity was reduced by 22% in ethanol-treated rats receiving PTU.

The increase in P450 content and in the rates of NDMA and ethanol oxidation in microsomes from ethanol-treated rats could be accounted for by an increase in CYP2E1 protein levels. Figure 6 shows an increase in immunoreactivity of a 52 kD protein band recognized by CYP2E1 anti-IgG in microsomes from chronic ethanol-treated rats. Densitometric analysis of CYP2E1 blots revealed a 3-fold increase in the relative intensity of CYP2E1 protein bands from ethanol-treated rat microsomes. CYP2E1 immunoreactivity in microsomes from PTU-treated rats did not differ from controls.

### 3.3.2 Chronic Acetone/PTU Study

Since chronic acetone treatment has been previously shown to induce microsomal CYP2E1 (Hetu and Joly, 1988; Koop and Casazza, 1985; Sakai et al., 1992), this regimen was employed to further extend our findings regarding the mechanism of action of PTU on microsomal metabolic activity in rats receiving ethanol chronically. Rats fed 1% (w/v) acetone in the drinking water for 14 days ingested an average daily dose of 90.8 ± 4.4 mg acetone/kg/day. Body weight gain was uniform between groups during the treatment period (Table 4). Chronic ingestion of acetone resulted in hepatomegaly as indicated by a 14% increase in liver weight. The increase in liver weight did not occur in acetone-treated rats receiving PTU. Intragastric administration of PTU (25 mg/kg/day) resulted in a substantial reduction in circulating thyroid hormone levels in both control and acetone treated-rats (Table 4). Serum levels of T4 and T3 were reduced by about 75% and 65%, respectively, after 14 days of treatment with PTU, but were unaffected by acetone ingestion.
Figure 6: Immunoblot analysis of CYP2E1 content in liver microsomes isolated from rats receiving (A) control diet (B) ethanol diet (C) control diet + PTU or (D) ethanol diet + PTU (n=7 per group). The left hand lane contained pre-stained protein standards with molecular weights as indicated. 6 μg of microsomal protein were separated electrophoretically in 7.5% SDS-polyacrylamide gels and immunoblotted with rabbit anti-rat CYP2E1 IgG. The rabbit anti-rat CYP2E1 IgG recognized a single 52 kD protein band corresponding to CYP2E1.
Table 4: Effects of chronic acetone and PTU treatments on body weight, liver weight and serum thyroid hormone levels.*

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Total T₄ (nmol/L)</th>
<th>Total T₃ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>241 ± 6</td>
<td>8.5 ± 0.4</td>
<td>62.6 ± 4.8</td>
<td>1.13 ± 0.08</td>
</tr>
<tr>
<td>Acetone</td>
<td>245 ± 7</td>
<td>9.7 ± 0.4†</td>
<td>53.4 ± 4.2†</td>
<td>1.01 ± 0.07†</td>
</tr>
<tr>
<td>Control + PTU</td>
<td>238 ± 3</td>
<td>8.4 ± 0.3‡</td>
<td>15.1 ± 0.1‡</td>
<td>0.40 ± 0.03‡</td>
</tr>
<tr>
<td>Acetone + PTU</td>
<td>240 ± 2</td>
<td>8.9 ± 0.3</td>
<td>15.1 ± 0.1‡</td>
<td>0.38 ± 0.02‡</td>
</tr>
</tbody>
</table>

* Rats were fed acetone in the drinking water (1% w/v) for 14 days, while controls received plain tap water only. One group of acetone-treated and control rats also received PTU (25 mg/kg/day) or vehicle by intragastric infusion, concurrently during the 14 day treatment period. Food was available *ad libitum*. Values are means ± SEM (n = 8 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by *post-hoc* Duncan's Test.

† p < 0.05, compared with the control group.
‡ p < 0.05, compared with the acetone-treated group.
Associated with the reduction in circulating thyroid hormone levels following PTU treatment, was a 40% decrease in microsomal CYP-reductase activity (as measured by the rate of cytochrome c reduction), which was observed in microsomes from rats receiving either PTU + acetone or PTU alone (Figure 7). CYP-reductase activity was not significantly altered by chronic acetone treatment.

Microsomal oxygen consumption was elevated 35% in microsomes from acetone-treated rats (Figure 8). Administration of PTU significantly reduced the rate of microsomal oxygen consumption in both acetone-treated (21%) and control rats (32%).

The microsomal generation of OH· (Figure 9), as determined by the formation of formaldehyde from DMSO, was not affected by acetone administration, but was significantly decreased by PTU treatment in both control rats (30%) and rats receiving acetone (21%). The effect of chronic acetone and PTU administration on microsomal P450 content and the metabolism of NDMA and ethanol are summarized in Table 5. Microsomes from acetone-treated rats showed an increase in total microsomal cytochrome P450 content (19%), and in the rate of ethanol oxidation (47%) and a greater than 2-fold increase in the rate of demethylation of NDMA to formaldehyde. PTU treatment also produced a significant increase in total P450 content in microsomes from both control and acetone-treated rats. The rate of microsomal NDMA metabolism was not affected by PTU treatment alone, however a small but significant increase resulted from PTU administration in rats receiving acetone. MEOS activity was reduced by 32% in ethanol- treated rats receiving PTU and by 25% in control rats receiving PTU.

3.4 Discussion

The results presented confirm previous findings that chronic administration of ethanol in a liquid diet induces hepatic microsomal CYP2E1 in rats (Lieber, 1988). In the present study,
Figure 7: Effect of PTU on NADPH-cytochrome P450 reductase (CYP-reductase) activity in liver microsomes from acetone-treated rats. PTU (25 mg/kg/day) was administered intragastrically to rats receiving either acetone or plain tap water concurrently for 14 days. CYP-reductase activity was determined by the rate of cytochrome c reduction at 550 nm. Vertical bars indicate mean ± SEM (n=7 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Figure 8: Effect of chronic acetone and PTU administration on the rate of NADPH-dependent oxygen consumption in rat liver microsomes. Rats were fed acetone or tap water for 14 days. PTU was administered concurrently at a dose of 25 mg/kg/day. Vertical bars indicate mean ± SEM (n=8 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Figure 9: Effect of chronic acetone and PTU treatments on the rate of microsomal production of hydroxyl radicals determined by the formation of formaldehyde from DMSO in the presence of 1.5 mM NADPH and 25 mM:50 mM ferric:EDTA. Vertical bars indicate mean ± SEM (n=8 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
Table 5: Effects of acetone and PTU treatments on hepatic microsomal P450 content and metabolism of ethanol (MEOS) and NDMA.*

<table>
<thead>
<tr>
<th></th>
<th>Total P450 (nmol/mg)</th>
<th>NDMA (nmol/mg/min)</th>
<th>MEOS (nmol/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.22 ± 0.05</td>
<td>0.96 ± 0.03</td>
<td>13.3 ± 0.4</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.45 ± 0.06†</td>
<td>2.36 ± 0.13†</td>
<td>19.6 ± 0.6†</td>
</tr>
<tr>
<td>Control + PTU</td>
<td>1.46 ± 0.11†</td>
<td>1.10 ± 0.07‡</td>
<td>9.0 ± 0.8‡‡</td>
</tr>
<tr>
<td>Acetone + PTU</td>
<td>1.75 ± 0.05†‡§</td>
<td>2.79 ± 0.19†‡§</td>
<td>15.7 ± 1.0‡§</td>
</tr>
</tbody>
</table>

* Rats were fed acetone in the drinking water (1% w/v) for 14 days, while controls received plain tap water only. One group of acetone-treated and control rats also received PTU (25 mg/kg/day) or vehicle by intragastric infusion, concurrently during the 14 day treatment period. Food was available ad libitum. Values are means ± SEM (n = 8 per group). Statistically significant differences between groups were determined by two-way analysis of variance followed by post-hoc Duncan's Test.

† p < 0.05, compared with the control group.
‡ p < 0.05, compared with the acetone-treated group.
§ p < 0.05, compared with the PTU-treated control group.
treatment with ethanol for 29 days resulted in increased levels of hepatic microsomal P450, a corresponding increase in the levels of CYP2E1 protein, as shown by Western blotting, and enhancement of CYP2E1 activity, as evidenced by elevated rates of ethanol oxidation (50%) and NDMA demethylation (100%). Accompanying the induction of CYP2E1 was a 70% increase in the rate of microsomal oxygen consumption and a 49% increase in the rate of OH· production. In agreement with previous studies (Hetu and Joly, 1988; Koop and Casazza, 1985; Sakai et al., 1992), chronic administration of acetone, a highly specific CYP2E1 substrate, also increased CYP2E1 catalytic activity. As was observed with ethanol, liver microsomes from acetone-treated rats displayed increased rates of oxygen utilization (35%), ethanol oxidation (47%) and NDMA demethylation (146%).

Increased oxygen consumption and oxygen radical formation by microsomes following chronic ethanol treatment are processes which have previously been implicated in promoting ethanol-induced liver injury and may be associated with elevated content or activity of CYP2E1 or CYP-reductase. CYP2E1 displays an exceptionally high degree of NADPH-oxidase activity (Gorsky et al., 1984; Ingelman-Sundberg and Johansson; 1984), generating H₂O₂ and H₂O and in both the absence and presence of substrates. Estimates derived from *in vitro* studies indicate that CYP2E1 *per se* can contribute significantly to hepatic oxygen consumption (see Thurman *et al.*, 1984a; 1984b; Tindberg and Ingelmar-Sundberg, 1989). Consequently, the induction of CYP2E1 oxidase activity by repeated ingestion of ethanol and the resulting increase in microsomal oxygen consumption would cause further depletion of hepatocellular oxygen and may lead to hypoxic injury to pericentral hepatocytes.

Increases in the levels of CYP-reductase may also account for the increase in microsomal oxygen consumption and oxygen radical formation in ethanol-treated rats. There is conflicting evidence regarding the effects of chronic ethanol and acetone treatments on CYP-reductase.
While increases in microsomal CYP-reductase activity have been reported in rats receiving chronic ethanol (Ekstrom and Ingelman-Sundberg, 1989; Joly et al., 1973; Lieber and DeCarli, 1970b; Shaw, 1989), other studies have failed to confirm this finding (French et al., 1983a; Badger et al., 1993a). Similarly, both increases (Sakai et al., 1992; Puntarulo and Cederbaum, 1988) and decreases in CYP-reductase activity (Hetu and Joly, 1988) have been reported in chronic acetone treated rats. Under our experimental conditions, chronic treatment with acetone had no effect on microsomal CYP-reductase activity. Microsomes from ethanol-treated rats displayed marginal increases in CYP-reductase activity (p = 0.06) and in the levels of CYP-reductase protein, indicating that ethanol may have a slight stimulatory effect on CYP-reductase. Hydroxyl radicals generated through the NADPH-dependent autoxidation of CYP-reductase in the presence of iron have been shown to contribute to ethanol oxidation by liver microsomes in vitro (Cederbaum, 1989; Winston and Cederbaum, 1983b). In our studies, Fe:EDTA (25 mM:50 mM) or EDTA (1 mM) was typically included in the reaction mixtures, which would likely promote the autoxidation of CYP-reductase and increase the extent of microsomal OH\(^-\) production. Thus, under our experimental conditions OH\(^-\) formation and MEOS activity may be determined largely by CYP-reductase activity. This could account for the finding that OH\(^-\) production was increased in ethanol-treated rats, where a marginal increase in CYP-reductase activity was observed, but was unaffected in acetone-treated rats, where CYP-reductase was clearly unaffected. The heightened contribution of CYP-reductase could also explain why the induction of MEOS activity following ethanol or acetone treatments was low in comparison to the 2-3 fold induction of NDMA activity (which is mainly dependent on CYP2E1 activity) and of CYP2E1 immunoreactivity.

In the present study administration of PTU, which inhibits both thyroid hormone synthesis and peripheral deiodination of thyroxine to triiodothyronine (Taurog, 1974; Yuki et al., 1982),
reduced serum T\textsubscript{3} and T\textsubscript{4} levels by 64% and CYP-reductase activity and protein levels by 27-34% and 66-69%, respectively. This observation is consistent with previous reports that reduction of thyroid hormone levels, by administration of antithyroid drugs such as PTU (Raheja \textit{et al.}, 1985) or methimazole (Ram and Waxman, 1992), or by surgical manipulations (Ram and Waxman, 1992; Rumbaugh \textit{et al.}, 1978; Waxman \textit{et al.}, 1989), lowers microsomal CYP-reductase levels and/or activity. In several respects, the effects of PTU reported here differ from antithyroid manipulations reported previously. A 50% decrease in CYP-reductase activity was observed in hypophysectomized female rats (Waxman \textit{et al.}, 1989), which is greater than the decrease in CYP-reductase observed in PTU-treated rats in our study and is probably attributable to the more complete antithyroid effect of hypophysectomy. In another relevant study by the same group (Ram and Waxman, 1992), the antithyroid drug methimazole produced a 92% reduction in T\textsubscript{4} levels (to levels near the detection limit) with a corresponding 67% decrease in CYP-reductase activity. In this case the greater decrease in CYP-reductase activity is probably again attributable to a more potent antithyroid effect of methimazole as compared to PTU.

Although PTU treatment produced a slight increase in microsomal P450 content, no effect was observed on CYP2E1 levels or catalytic activity, as demonstrated by Western blots and rates of NDMA demethylation, respectively. The possible identity of the other P450 isoforms induced by PTU was not examined in the present study, however the steroid hydroxylases CYP3A2 and CYP2A2 are likely candidates (Waxman \textit{et al.}, 1989). In previous studies hypophysectomy was shown to increase the hepatic expression of CYP3A2, an effect which was abolished by T\textsubscript{4} replacement (Ram and Waxman 1991), and to increase the levels of CYP3A2 and CYP2A2 protein (Waxman \textit{et al.}, 1989).

PTU has been previously employed both in clinical (Orrego \textit{et al.}, 1987) and experimental studies (Israel \textit{et al.}, 1975\textit{a}) to revert or prevent ethanol-induced liver injury. The results of this
study implicate several mechanisms which may contribute to the hepatoprotective effect of PTU. First, PTU was found to offset the increase in microsomal oxygen consumption associated with CYP2E1 induction in ethanol- and acetone-treated rats. This observation is consistent with studies indicating that PTU administration in chronic ethanol-fed rats reduces hepatic oxygen utilization both in isolated perfused livers (Rachamin et al., 1985; Thurman et al., 1984a; 1984b), and in the intact animal (Carmichael et al., 1993). Secondly, in contrast to ethanol which increased OH· production, PTU treatment reduced the rate of OH· formation in ethanol- and acetone-treated rats. Lastly, microsomal oxidation of ethanol to acetaldehyde, which was substantially increased following treatment with ethanol or acetone, was greatly reduced in microsomes from PTU-treated rats.

The observed reduction in microsomal oxygen consumption, OH· production and ethanol oxidation in PTU-treated rats appears to result from a decrease in the levels and activity of CYP-reductase. This may have a dual effect involving either a reduction in electron transport for CYP2E1 oxidase reactions, or alternatively, a decrease in the iron-catalyzed autoxidation of CYP-reductase, occurring independently of CYP2E1. Our results indicating a decreased rate of ethanol oxidation in PTU-treated rats, in the absence of changes in CYP2E1 levels or catalytic activity, can be explained by the reduction in iron-catalyzed OH· production, due to the lower levels and activity of CYP-reductase. The role of iron in these reactions is complex and depends on the chelated form of iron utilized, the concentration used and the oxygen radical being investigated (Cederbaum, 1992). Although our system contained EDTA and trace amounts of iron it is possible that similar conditions may be reached in the physiological state. Previous studies have shown that both Fe:ATP and Fe:citrate, which are more likely to exist in vivo, are also capable of promoting OH· production and lipoperoxidation (Cederbaum, 1989). In vitro, microsomal iron levels as low as 1 μM are sufficient to catalyze Haber-Weiss reactions leading to NADPH-
dependent OH\(^{-}\) formation by CYP-reductase (Winston and Cederbaum, 1983a). The extent to which CYP-reductase contributes to hepatic oxygen consumption, oxygen radical generation and ethanol oxidation \textit{in vivo} is unknown, but it may be significant under conditions in which liver iron levels are elevated, such as in chronic ethanol-fed rats (Shaw, 1989) and in human alcoholics with liver disease (Williams \textit{et al.}, 1967; Zimmerman \textit{et al.}, 1961). This view is in line with recent findings that coadministration of iron in rats receiving chronic ethanol treatment hastens the onset and increases the severity of ethanol-induced liver injury (Stal and Hultcrantz, 1993; Tsukamoto \textit{et al.}, 1992).

In summary, these results indicate that chronic ethanol ingestion results in the induction of CYP2E1 and enhances microsomal oxygen utilization, OH\(^{-}\) production and ethanol oxidation; processes which may be involved in promoting ethanol-induced liver pathologies. The ability of PTU to minimize these effects by lowering CYP-reductase levels may contribute to its therapeutic efficacy in reducing liver injury.
4. STUDY 2

EFFECT OF PROPYLTHIOURACIL AND GRANULOCYTE COLONY STIMULATING FACTOR ON CIRCULATING NEUTROPHILS AND LIVER INJURY IN RAT MODELS OF EXPERIMENTAL ALCOHOLIC LIVER DISEASE.

4.1 Introduction

The accumulation of neutrophils in infected tissues is essential for the defense against invading microorganisms, however neutrophil-derived oxidants and proteolytic enzymes can also promote injury to surrounding healthy tissue. A growing body of evidence suggests that neutrophils may play an important role in mediating ethanol-induced liver injury. Alcoholic hepatitis in humans is characterized by both an increase in the number of neutrophils in the circulation (Craper, 1983; Hill et al., 1993; Khortus et al., 1991; Lischner et al., 1971) and by marked neutrophil infiltration of the liver (Takahashi et al., 1987; International Group, 1987). Several factors involved in the regulation of chemotaxis, the process by which neutrophils migrate from the circulation to sites of inflammation, appear to be altered during the progression of alcoholic liver disease. The cytokines interleukin-1 and interleukin-8, which are involved in the regulation of neutrophil mobilization and tissue adhesion, are significantly elevated in the blood of patients with alcoholic hepatitis (Hill et al., 1993; Sheron et al., 1993). Furthermore, hepatocytes metabolizing ethanol generate metabolic byproducts that stimulate neutrophil chemotaxis (Shiratori et al., 1992a; Shiratori et al., 1992b; Roll et al., 1989). These effects in combination with ethanol-induced leukocytosis, may contribute to an exaggerated inflammatory response and consequent liver injury. An increase in the number of circulating neutrophils may therefore be an important initiating event leading to neutrophil infiltration of the liver and subsequent tissue injury.

Portions of this chapter were published previously by the author (Ross et al., 1998b).
The antithyroid drug propylthiouracil (PTU) has been shown to markedly reduce the risk of mortality from alcoholic liver disease in humans (Orrego et al., 1987), and protects against a variety of other forms of inflammatory liver injury in rats (Israel et al., 1975; Britton et al., 1985; Yamada et al., 1981; Linscheer et al., 1980; Raheja et al., 1982; Orrego et al., 1986; Oren et al., 1996; Cooper et al., 1984), however the underlying mechanisms are not fully understood. PTU treatment often induces transient leukopenia (Wing and Aspen, 1952; Amrhein et al., 1970; Kariya et al., 1983; 1984), typically involving a reduction in the number of circulating neutrophils, and in rare cases may induce agranulocytosis, a condition characterized by a massive reduction in neutrophil counts (Trotter, 1962; Rosove, 1977; Cooper et al., 1983). PTU-induced neutropenia could counteract the increase in circulating neutrophils and the subsequent infiltration of neutrophils in the liver associated with ALD and may represent an important mechanism contributing to the drug’s hepatoprotective effects.

The present study investigated the relationship between circulating neutrophils and liver injury in rats receiving chronic ethanol and examined the effect of PTU on circulating neutrophil counts in normal and ethanol-fed rats. Since the degree of ethanol-induced liver injury varies considerably according to the method of ethanol administration, we studied the effect of ethanol liquid diets given by both the (a) oral feeding and (b) continuous intragastric infusion methods. The oral diet feeding method, based on that of Lieber and DeCarli (1982), induces relatively minor liver injury, while the intragastric infusion model is reported to produce a pattern of liver damage which more closely resembles the hepatitis and fibrosis characteristic of human alcoholic liver disease (Tsukamoto et al., 1985). A further objective of this study was to determine whether an artificially induced increase in circulating neutrophil counts could exacerbate ethanol-induced liver injury in the Lieber-DeCarli model. This was examined in ethanol-treated rats following
administration of human granulocyte colony-stimulating factor (G-CSF - Neupogen®), a recombinant analog of an endogenous glycoprotein that selectively regulates the proliferation and differentiation of neutrophil progenitor cells (Hollingshead and Goa, 1991).

4.2 Method

4.2.1 Oral Ethanol Feeding Model

Male Wistar rats (Charles River, Quebec) with an initial body weight of 125-135 g were given continuous access for 30 days to a liquid diet containing ethanol, or an isocaloric control diet. Diets were delivered by an automated feeding system to ensure equal consumption between groups, as described by Israel et al. (1984). Liquid diets had a total caloric content of 1 kcal/ml with 32% of total calories supplied as fat (a mixture of 80% corn oil, 12% olive oil and 8% cod liver oil), 9% as carbohydrates (maltose dextrin), 23% as protein (casein) and 36% as ethanol or additional carbohydrates. Diets also contained 4.0 g/L vitamin mix No. 20315 (Bioserv, Frenchtown, NJ), 10.03 g/L Bioserv salt mix No. 711, 4 g/L viscarin, 269 mg/L DL-methionine and 228 mg/L choline chloride. The exact diet formulations are listed in Appendix 1.

4.2.2 Continuous Intragastric Ethanol Infusion Model

Male Wistar rats (Charles River, Quebec) with an initial body weight of 310 - 325 g received continuous infusion of liquid diets for 87 days through permanently implanted gastric catheters (Tsukamoto et al., 1985). Liquid diets had a caloric content of 1 kcal/ml consisting of 35% of calories as fat (corn oil), 25% as protein, 32% as ethanol and 8% as carbohydrates (Tsukamoto et al., 1985; French et al., 1985). Diets also contained 0.25% (w/v) carbonyl iron, which has been reported to potentiate ethanol-induced liver injury (Tsukamoto et al., 1992). Control rats received diets as above with isocaloric carbohydrates substituted for ethanol. Ethanol
infusion was started at a dose of 8 g/kg/day and gradually increased to 14 g/kg/day as metabolic tolerance developed, such that urinary ethanol concentrations were maintained within a range of 200-400 mg/dL throughout the experiment. Urine was collected daily at 9:00 am and ethanol concentrations were determined by gas chromatography. Tail vein blood samples were collected on days 24, 58 and 72 for the determination of serum alanine aminotransferase (ALT) concentrations as described below.

4.2.3 Propylthiouracil Treatment

The effect of PTU was examined in rats receiving liquid diets by the continuous intragastric infusion method. In the first phase of the study, control liquid diets were infused continuously and PTU was administered daily by gavage for 4 days at a dose of 1 mg/kg. The dose of PTU was then increased to 5, 25 and 50 mg/kg over successive four day intervals. In the second phase, PTU treatment was discontinued and rats were switched from control diets to ethanol-containing diets for 2 weeks. PTU treatment was then repeated over successive 4 day intervals at escalating doses of 1, 5 and 25 mg/kg. An additional group of rats (controls) received an equivalent dose of vehicle (water) instead of PTU throughout the control diet and ethanol-diet treatment phases.

4.2.4 G-CSF Treatment

Two groups of rats receiving ethanol-containing or control diets by oral feeding were treated with human recombinant G-CSF (Neupogen® - Amgen Inc., Thousand Oaks, CA). G-CSF at a dose of 100 μg/kg/day (s.c.) or an equal volume of vehicle (10 mM sodium acetate, 5% mannitol and 0.004% Tween 80 - pH 4.0) was administered daily during the final 4 days of ethanol feeding. Following G-CSF treatment, tail vein blood samples were collected for the
determination of serum ALT.

### 4.2.5 Blood Biochemistry and Cell Count

Blood samples were collected from the tail vein and either mixed with lithium heparin for differential blood cell counts (Vitatech Diagnostics, Missasauga, Canada) or allowed to clot at room temperature, after which serum was separated by centrifugation at 2500 x g for 10 minutes and stored at -70°C. The concentration of ALT in serum samples was analyzed using a diagnostic kit (DG159-UV) from Sigma Chemicals (St. Louis, MO).

### 4.2.6 Liver Histology

On the final day of experiments, rats were anesthetized with ether, and the livers were removed, rinsed and weighed. A portion of the right median lobe was fixed in 10% neutral buffered formalin and stained for light microscopy with hematoxylin and eosin. Coded liver samples were examined by a pathologist and the severity of injury was assessed as follows: steatosis (the percentage of liver cells containing fat) 1 = 0-25% of cells containing fat, 2 = 26-50%, 3 = 51-75%, 4 = >75%. Inflammation and necrosis were scored as: 0 = no foci, 1 = one focus per low power field, 2 = 2 or more foci. At least 10 fields (100 x magnification) were examined per liver sample. The pathology score was calculated as the sum of the individual parameter scores (Nanji et al., 1993).

### 4.2.7 Statistics

Group data are presented as the mean ± SEM. Statistical differences between groups were calculated by Student’s unpaired t-test, or for multifactorial comparisons, by ANOVA followed by Duncan's post-hoc tests. Results were considered significant at p < 0.05.
4.3 Results

4.3.1 Oral Ethanol Feeding

Rats consuming ethanol-containing liquid diets orally received an average ethanol dose of 12 g/kg body weight/day during the 30 days of treatment. Final body weights were similar in both ethanol-fed (237 ± 11) and pair-fed (239 ± 7 g) groups. The hematological effects of ethanol in these rats are summarized in Table 6. Circulating neutrophil counts were not affected by ethanol treatment, however small but significant increases in monocyte count, erythrocyte hemoglobin content and hematocrit were evident. The concentration of ALT in the serum of rats receiving ethanol orally was elevated by 79% as compared to pair-fed controls (Figure 10A). Liver mass was increased slightly (15%) but significantly (Figure 10B). Histopathological examination of liver specimens from ethanol-fed rats revealed a minor but significant degree of liver injury as indicated by the pathology score (Figure 10C). Slight to moderate steatosis (<50%) throughout the lobule was evident in the majority of these rats, but no signs of hepatocyte necrosis, fibrosis or inflammatory cell infiltrates were evident. Livers from pair-fed rats did not show any significant morphological abnormalities.

4.3.2 Intragastric Ethanol Infusion

Rats receiving ethanol diets by intragastric infusion had an average daily urinary ethanol concentration of 353 ± 15 mg/dL during the 87 days of ethanol treatment. Final body weights in ethanol-treated rats (441 ± 8 g) were slightly lower than in pair-fed controls (477 ± 9 g), despite the fact that each group had equal caloric intake. A similar reduction in the rate of weight gain in rats receiving intragastric ethanol has been reported previously (French et al., 1984). Hematological parameters in rats receiving liquid diets intragastrically are shown in Table 7. Circulating neutrophils counts in ethanol-infused rats were elevated approximately twofold in
Table 6. Hematological parameters in rats fed ethanol-containing or isocaloric control liquid diets orally for 30 days.

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes</th>
<th>Erythrocytes</th>
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<tbody>
<tr>
<td></td>
<td>Total WBC (x 10^9/l)</td>
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<tr>
<td></td>
<td>Neutrophils (x 10^9/l)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes (x 10^9/l)</td>
<td></td>
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<tr>
<td></td>
<td>Monocytes (x 10^9/l)</td>
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<tr>
<td></td>
<td>Eosinophils (x 10^9/l)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RBC (x 10^12/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hematocrit (%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hb (g/l)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCV (fl)</td>
</tr>
<tr>
<td>Pair-fed Control</td>
<td>6.9 ± 1.0</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.2 ± 0.3</td>
<td>39 ± 1</td>
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<tr>
<td></td>
<td>5.5 ± 0.9</td>
<td>137 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.2 ± 0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Ethanol-fed</td>
<td>6.9 ± 0.5</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1.1 ± 0.2</td>
<td>43 ± 1†</td>
</tr>
<tr>
<td></td>
<td>5.4 ± 0.4</td>
<td>145 ± 2†</td>
</tr>
<tr>
<td></td>
<td>0.4 ± 0.1†</td>
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<td></td>
<td>0.1 ± 0.0</td>
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</table>

Data are expressed as mean ± SEM for 6 rats per group.

† denotes statistically significant differences between groups as determined by Student's t-test.
Figure 10: Effect of oral ethanol feeding on indices of liver injury in rats. Ethanol-containing or isocaloric control liquid diets were administered for 30 days prior to assessment of serum alanine aminotransferase (ALT) concentration (A), liver to body weight ratio percentage (B) and the histological severity of liver injury as reflected by the pathology score (C). Vertical bars represent mean ± SEM (n=6 per group). * indicates significant difference between groups as determined by Student's t-test.
Table 7. Hematological parameters in rats receiving ethanol-containing or isocaloric control liquid diets by intragastric infusion for 87 days.

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes</th>
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<th>Erythrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total WBC</td>
<td>Neutrophils (x 10^9/l)</td>
<td>Lymphocytes (x 10^9/l)</td>
</tr>
<tr>
<td><strong>Pair-fed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20.9 ± 2.9</td>
<td>9.8 ± 1.7</td>
<td>8.8 ± 0.8</td>
</tr>
<tr>
<td>Ethanol-fed</td>
<td>26.8 ± 4.7</td>
<td>18.4 ± 2.7†</td>
<td>5.6 ± 1.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM for 3-9 rats per group.
† denotes statistically significant differences between groups as determined by Student's t-test.
comparison to control rats. Other significant hematological alterations associated with ethanol infusion included increases in the number of circulating monocytes and eosinophils. All other hematological parameters were comparable between the two groups. Biochemical and histological indices indicated significant liver injury in rats receiving intragastric ethanol. The concentration of ALT in the serum of rats receiving ethanol infusion increased progressively over the duration of the experiment as shown in Figure 11A. Maximal elevation in serum ALT was observed on day 72, when ALT levels in ethanol-infused rats were 163% higher than those in control rats. Severe hepatomegaly was also evident in ethanol-infused rats, which had liver to body weight ratios roughly twice those of control rats (Figure 11B). Liver biopsies showed significant liver injury in ethanol-infused rats as reflected by the higher pathology scores (Figure 11C). Liver injury was characterized mainly by severe steatosis throughout the lobule. A few isolated necrotic hepatocytes and mitotic figures were also observed, however widespread necrosis and neutrophil infiltration were not evident.

4.3.3 Propylthiouracil Treatment

Treatment with PTU during 4 day intervals at escalating doses of 1-50 mg/kg (p.o.) did not significantly affect circulating neutrophil counts in rats receiving continuous intragastric infusion of control-liquid diets (Figure 12). To determine whether the response to PTU may be altered in ethanol-treated rats, the same rats were given ethanol-containing liquid diets by infusion for 2 weeks, followed by treatment with PTU at escalating doses of 1, 5 and 25 mg/kg for 4 days per dose. No significant difference in neutrophil counts was observed between PTU-treated and vehicle-treated rats receiving ethanol (Figure 13).
Figure 11: Effect of chronic intragastric ethanol infusion on indices of liver injury in rats. Rats received ethanol-containing (n=3) or isocaloric control liquid diets (n=9) continuously by intragastric infusion for 87 days. Tail vein blood samples were collected on days 24, 58 and 72 for the determination of serum alanine aminotransferase (ALT) concentrations (A). Liver to body weight ratio percentage (B) and the liver histopathology score (C) were determined post-mortem. Vertical bars represent mean ± SEM. Statistically significant differences between groups were determined by Student's t-test (B and C) or, for multigroup comparisons (C), by ANOVA and Duncan's post-hoc tests.

* indicates significant difference (p<0.05) as compared to corresponding control group.
Figure 12: Effect of propylthiouracil (PTU) on circulating neutrophil counts in rats receiving continuous intragastric infusion of control liquid diets. PTU was administered at escalating doses of 1, 5, 25 and 50 mg/kg/day (p.o.) for four days at each dose. Control rats received an equivalent volume of vehicle throughout the treatment period. Vertical bars represent mean ± SEM (n=3-5 per group). Statistically significant differences between groups were determined by ANOVA and Duncan's post-hoc tests.
Figure 13: Effect of propylthiouracil (PTU) on circulating neutrophil counts in rats receiving continuous intragastric infusion of ethanol-containing liquid diets. Rats received ethanol-containing liquid diets for two weeks followed by PTU treatment at escalating doses of 1, 5 and 25 mg/kg/day (p.o.) for four days at each dose. Control rats received an equivalent volume of vehicle throughout the treatment period. Vertical bars represent mean ± SEM (n=3-5 per group). Statistically significant differences between groups were determined by ANOVA and Duncan's post-hoc tests.
4.3.4 G-CSF Treatment

Daily treatment with G-CSF (100 μg/kg/day) for 4 days in rats receiving ethanol by oral feeding resulted in a significant increase in the total number of circulating neutrophils and monocytes in both ethanol- and pair-fed rats (Table 8). Neutrophil counts were elevated 6-fold in pair-fed control rats treated with G-CSF and 9-fold in rats receiving ethanol and G-CSF. Although G-CSF appeared to have a greater effect on neutrophil counts in ethanol-treated rats, the interaction was not statistically significant. Other hematological parameters were not affected by G-CSF treatment. The increase in circulating neutrophils was not associated with an increase in the biochemical indices of liver injury. G-CSF treatment had no effect on the serum levels of ALT in either ethanol- fed or control groups (Figure 14A). Liver to body weight ratios were also unaltered following administration of G-CSF in either ethanol-fed or control rats (Figure 14B).

4.4 Discussion

4.4.1 Neutrophils in Rat Models of Alcoholic Liver Disease

Neutrophils are thought to play an important role in the pathogenesis of alcohol-induced liver injury. In this study we report a relationship between the number of circulating neutrophils and the severity of alcohol-induced liver injury in rats. The effect of chronic ethanol on circulating neutrophil counts was dependent on the alcohol administration paradigm employed. Rats receiving ethanol by oral diet feeding for 30 days exhibited steatosis, hepatomegaly and elevated serum ALT consistent with minor liver injury, but exhibited no alterations in the number of circulating neutrophils. Liver injury in rats receiving ethanol diets for 87 days using the continuous intragastric infusion method was moderate and was accompanied by marked neutrophil leukocytosis. The greater severity of liver injury observed with the intragastric infusion
Table 8: Effect of daily treatment with G-CSF (100 μg/kg/day) on hematological parameters in rats fed ethanol-containing or isocaloric control liquid diets orally for 30 days.

<table>
<thead>
<tr>
<th>Leukocytes</th>
<th>Erythrocytes</th>
<th>Hemat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total WBC (x 10^9/l)</td>
<td>Neutrophils (x 10^9/l)</td>
</tr>
<tr>
<td>Control Diet</td>
<td>7.5 ± 1.1</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>Ethanol Diet</td>
<td>7.9 ± 0.9</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>Control Diet + G-CSF</td>
<td>18.5 ± 2.0†‡</td>
<td>9.6 ± 1.8†‡</td>
</tr>
<tr>
<td>Ethanol Diet + G-CSF</td>
<td>22.8 ± 0.8†‡</td>
<td>13.5 ± 1.1†‡</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM for 6-7 rats per group.

Statistically significant differences between groups as determined by ANOVA and Duncan's post-hoc tests are indicated as:

† p < 0.05 compared to control group.
‡ p < 0.05 compared to ethanol-treated group.
* p < 0.05 compared to G-CSF treated control group.
Figure 14: Effect of granulocyte colony-stimulating factor (G-CSF) on indices of liver injury in chronic ethanol-fed rats. Rats received ethanol-containing or control diets by oral feeding for 30 days. G-CSF (100 μg/kg/day) or vehicle was administered (s.c.) during the final 4 days of ethanol feeding. Following G-CSF treatment, tail vein blood samples were collected for the measurement of serum ALT (A) and liver to body weight ratio percentage was determined (B). Statistical differences between the treatment groups were not significant, as determined by two-way analysis of variance.
method is presumably due to a combination of the higher blood alcohol concentrations achieved and the presence of carbonyl iron in the liquid diets (Tsukamoto et al., 1992; Tsukamoto et al., 1990). The pattern of liver injury associated with intragastric ethanol infusion consisted mainly of severe steatosis and scattered hepatocyte necrosis. Fibrotic lesions were not evident in any of the biopsies examined. Despite the increase in circulating neutrophils, extensive inflammatory infiltration (i.e., neutrophils and/or monocytes) was not typically observed in the liver biopsies. These histopathological features differ from those commonly observed in human alcoholic hepatitis and in some rat models following long-term (i.e., >3 months) intragastric ethanol infusion (Tsukamoto et al., 1992; Tsukamoto et al., 1990). The present observation that liver injury occurs in the absence of extensive inflammatory cell infiltration suggests that ethanol-induced leukocytosis may not have directly contributed to the liver damage observed in our intragastric ethanol infusion model.

The involvement of circulating neutrophils in the pathogenesis of alcohol-induced liver injury was investigated further by administering G-CSF to rats receiving ethanol using the Lieber-DeCarli model. Repeated administration of G-CSF in rats after 30 days of oral ethanol diet feeding resulted in a 9-fold increase in the number of circulating neutrophils, but did not increase liver injury induced by ethanol treatment. The direct involvement of circulating neutrophils in hepatic injury following chronic ethanol administration in rats is unlikely based on the findings that: (a) G-CSF greatly increased circulating neutrophil counts, but did not potentiate ethanol-induced liver injury; and (b) neutrophil infiltration of the liver was minimal following ethanol treatment even in the presence of substantially elevated circulating neutrophil counts.

In summary a two-fold increase in circulating neutrophils was associated with liver injury in the Tsukamoto and French model of ethanol administration. However a 6-9 fold increase in circulating neutrophils, following treatment with G-CSF, did not result in liver damage in the
Lieber-DeCarli model. These studies indicate that circulating neutrophils are not a major determinant of alcoholic liver disease.

4.4.2 Neutropenia as a Mechanism of Propylthiouracil Hepatoprotection?

Abnormal reductions in circulating neutrophil counts have been previously reported in PTU-treated rats (Kariya et al., 1983; 1984) and in humans during therapy for hyperthyroidism (Wing and Aspen, 1952; Amrheim et al., 1970; Trotter, 1962; Rosove, 1977; Cooper et al., 1983). We postulated that this effect may prevent neutrophil-mediated tissue damage and could therefore contribute to the ability of PTU to prevent inflammatory liver injury induced by ethanol and other toxins. In the present study, PTU did not affect circulating neutrophil counts in rats receiving intragastric infusion of either ethanol-containing or control liquid diets. This result differs from those of Kariya et al. (1983; 1984), however the dose of PTU used (255 mg/kg/day i.p.) was considerably higher than that used in the present study (1-50 mg/kg/day p.o.) and well in excess of the doses typically used in the treatment of ALD (Orrego et al., 1987). Based on the failure of PTU to induce neutropenia at therapeutically relevant doses in both control and ethanol-treated rats, it can be concluded that this is not a mechanism contributing to the hepatoprotective effect of PTU against ethanol-induced liver injury described in prior studies.
5. STUDY 3

EFFECT OF ANTITHYROID DRUGS ON HYDROXYL RADICAL FORMATION AND α-1-PROTEINASE INHIBITOR INACTIVATION BY NEUTROPHILS: THERAPEUTIC IMPLICATIONS.⁷

5.1 Introduction

Propylthiouracil, a drug widely used in the treatment of hyperthyroidism, has also been shown to protect against various forms of inflammatory liver injury in rats, including that induced by hypoxia-reoxygenation in ethanol-fed animals (Israel et al., 1975a), acetaminophen (Raheja et al., 1982), carbon tetrachloride (Orrego et al., 1976), thioacetamide (Oren et al., 1996) and galactosamine (Cooper et al., 1984). In clinical studies propylthiouracil resulted in a 50-60% reduction in mortality rates from alcoholic liver disease (Orrego et al., 1987). However, subsequent use of propylthiouracil in the treatment of alcoholic liver disease has been limited due mainly a lack of understanding regarding the mechanism of drug action.

A common finding in the aforementioned conditions is the presence of neutrophilic infiltrates in the liver. Reactive oxygen species and proteolytic enzymes are released by activated neutrophils during the respiratory burst, both of which have been proposed to play an important role in mediating the cell injury associated with inflammation (Smith, 1994). The formation of the highly toxic hydroxyl radical may play an important role in neutrophil-mediated tissue injury, as suggested by the observation that hydroxyl radical scavengers can protect target tissues from neutrophil-mediated damage in vitro and in vivo (Ward et al., 1983; Fox, 1984). NADPH oxidase (NADPH-FMN oxidoreductase: E.C. 1.6.99.1), an enzyme present in the neutrophil membrane, catalyzes the formation of superoxide which undergoes spontaneous and superoxide dismutase-

⁷ Ross et al., 1998a
catalyzed transformation to hydrogen peroxide, resulting in the formation of hydroxyl radical by the iron catalyzed Haber-Weiss reaction. The relevance of the latter reaction and a possible alternative mechanism for hydroxyl radical formation is considered in the Discussion (see 5.4). Hicks and coworkers (1992) showed that propylthiouracil has a powerful antioxidant action, scavenging hydroxyl radicals and preventing lipoperoxidation, and proposed that propylthiouracil could protect against liver injury by such a mechanism.

An alternative mechanism is also conceivable. Propylthiouracil (PTU) and methimazole (MMI), a related antithyroid drug, have been shown in vitro to inhibit myeloperoxidase (EC 1.11.1.7) (Taurog and Dorris, 1992), an enzyme present in neutrophil azurophilic granules that catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions. Recent studies indicate that neutrophil myeloperoxidase is a member of the same gene superfamily as thyroid peroxidase (Kimura and Ikeda-Saito, 1988), which iodinates thyroglobulin, a precursor of thyroid hormones. Hypochlorous acid oxidizes a number of biochemical targets (Arnhold et al., 1991), including the proteinase inhibitor α-1-antitrypsin (or α-1-proteinase inhibitor: A1PI). A1PI is generated by the liver and released into the plasma where it rapidly and irreversibly inactivates neutrophil-derived serine proteases such as elastase (Travis and Salvesen, 1983), which can otherwise degrade extracellular structural proteins and induce injury to host tissue (Gadek and Crystal, 1983; Wong and Travis, 1980).

Recent studies have suggested that hypochlorous acid generated by myeloperoxidase may contribute to the production of hydroxyl radical by a reaction that does not require the presence of a transition metal catalyst (1) (Candeias et al., 1993; Ramos et al., 1992). However, the relative contribution of this mechanism to the overall formation of hydroxyl radicals is not clear.

\[ \text{HOCl} + \text{O}_2^- \rightarrow \text{OH}^- + \text{O}_2 + \text{Cl}^- \] (1)
Since the generation of hypochlorous acid by myeloperoxidase may lead to either hydroxyl radical formation or to A1PI inactivation, an inhibitory effect of antithyroid drugs on myeloperoxidase might contribute a dual mechanism in the protection against tissue injury. The purpose of the present study was to determine the effect of therapeutically relevant concentrations of antithyroid drugs on the neutrophil-mediated inactivation of A1PI and on the formation of reactive oxygen species (superoxide, hypochlorous acid and hydroxyl radicals) in rat neutrophils. A second objective was to determine the relative contribution of myeloperoxidase to the generation of hydroxyl radicals by neutrophils. For this purpose we have also utilized chicken neutrophils, which are naturally devoid of myeloperoxidase (Rausch and Moore, 1975), to study the formation of reactive oxygen species.

5.2 Method

5.2.1 Reagents

α-1-proteinase inhibitor (A1PI), ammonium chloride, catalase (38000 U/mg protein- from bovine liver), desferrioxamine mesylate, disodium ethylenediaminetetraacetic acid (EDTA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), diethylenetriaminepentaacetic acid (DTPA), ferricytochrome c (from horse heart), heparin (176 U/mg protein - from porcine intestinal mucosa), methimazole, phorbol 12-myristate 13-acetate (PMA), porcine pancreatic elastase (52.8 U/mg protein), propylthiouracil, 4-pyridyl 1-oxide N-tert-butyl nitrotrone (4-POBN), sodium carbonate monohydrate (KHCO₃), N-succinyl-L-(ala)₁₃-p-nitroanilide (SLAPN), superoxide dismutase (4000 U/mg protein, from bovine erythrocytes), taurine and trypan blue (0.4%) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's phosphate buffered saline (d-PBS), Hanks balanced salt solution (HBSS) without Ca⁺⁺, Mg⁺⁺ or phenol red, and Tris HCl were purchased from Gibco, dextran T-500 and Percoll, from Pharmacia, [1-¹³C]ethanol from Aldrich.
(St. Louis, MO), Chelex-100 chelating resin from Bio-Rad Laboratories (Richmond, CA), EDTA-coated Vacutainer tubes from Becton Dickinson (Franklin Lakes, NJ) and 100% ethanol from RDL Alcohols. Diphenylene iodonium chloride (Biomol Research Laboratories, Plymouth Meeting, PA) and PMA were prepared in dimethylsulfoxide (Fisher, Fair Lawn, NJ) as 1 mg/ml stock solutions and stored at -20°C. HBSS was pretreated with Chelex-100 resin to remove residual iron contamination according to the method of Buettner (1988). Sterile stock solutions of Percoll were prepared weekly in 10x d-PBS, stored at 4°C and diluted to working concentrations with 1x d-PBS (pH 7.4). 5-thio-2-nitrobenzoic acid (TNB) was synthesized from DTNB according to the method of Riddles et al. (1983). DTNB (0.5 M) was dissolved in 50 mM potassium phosphate buffer (pH 7.4) and titrated to pH 12 with 10 N NaOH. After 5 minutes, the pH was lowered to 7.4 with 10 N HCl, yielding a 1 mM solution of TNB.

5.2.2 Rat Neutrophil Isolation

Male Sprague Dawley rats were anesthetized with sodium pentobarbital and 15 ml of blood was collected via the abdominal aorta into a 20 ml syringe with 1.5 ml of heparin (100 U/ml final) in 0.9% saline and 2 ml of 6% dextran T-500 in 0.9% saline. The mixture was sedimented in the syringe for 1 hour at room temperature. The leukocyte-rich buffy coat was expelled into a 50 ml polypropylene tube, mixed with cold d-PBS and centrifuged at 1200 g for 1 hour at 4°C. The supernatant was discarded and the pelleted cells were resuspended in 10 ml d-PBS. Neutrophils (polymorphonuclear leukocytes: PMN) were further purified on a discontinuous Percoll gradient (Szucs et al., 1994). The cell suspension was carefully layered onto a double density gradient, consisting of 10 ml of 65% Percoll over 10 ml of 89% Percoll, and centrifuged at 1200 g for 1 hour at 25°C. After separation the neutrophils were aspirated from the gradient interface, resuspended in 40 ml cold d-PBS and centrifuged at 1200 g for 20 minutes at
4°C. To lyse residual erythrocytes, the resulting pellet was suspended in 3 ml of ice cold 155 mM ammonium chloride solution containing 10 mM KHCO₃ and 0.1 mM EDTA, pH 7.4 (Roos and De Boer, 1986). After a 5 minute incubation on ice, the suspension was diluted in d-PBS and centrifuged at 1200 g for 20 minutes at 4°C. The pellet was washed twice in 40 ml of cold d-PBS, resuspended in HBSS at a concentration of 1.5-2.5 x 10⁸ cells/ml and kept on ice prior to the experiments. Neutrophil suspensions contained > 90% neutrophils and viability was > 95%, as determined by trypan blue exclusion.

5.2.3 Chicken Neutrophil Isolation

Female white leghorn chickens (Buckshire Farms, Perkasie, PA) aged 21 to 23 weeks were anesthetized with isoflurane and approximately 35 ml of blood was collected into EDTA-coated Vacutainer tubes via a jugular vein catheter. Omitting the dextran sedimentation step, the blood was diluted 1:1 with d-PBS, layered onto Percoll gradients and processed using the same procedure described above for rats.

5.2.4 Hypochlorous Acid Determination

The production of hypochlorous acid was determined from the formation of taurine chloramine and the subsequent oxidation of TNB to DTNB (Weiss et al., 1982). Eppendorf tubes containing rat or chicken neutrophils (5 x 10⁶ cells/ml), taurine (20 mM) and PMA (100 ng/ml) were incubated for 30 minutes at 37 °C. The reaction was stopped by the addition of catalase (50 μg/ml) and the tubes were placed on ice for 10 minutes. Cells were removed by centrifugation at 14000 x g at 4°C for 5 minutes. 800 μl of supernatant was combined with 200 μl of TNB (0.2 mM final) and incubated in the dark at room temperature for 10 minutes. The concentration of DTNB in duplicate samples was determined spectrophotometrically from the loss of absorbance at
412 nm using an extinction coefficient of 13.6 mM\(^{-1}\) cm\(^{-1}\) (Ellman, 1959). Inhibitors were added as described below for \(O_2^-\) measurements.

5.2.5 Superoxide Determination

The production of superoxide was determined from the superoxide dismutase-inhibitable reduction of ferricytochrome c (Markert et al., 1984). Reaction mixtures contained rat or chicken neutrophils (2 x 10\(^6\)/ml), cytochrome c (80 \(\mu\)M) and PMA (100 ng/ml) in HBSS in a final volume of 1 ml. Samples were incubated for 30 minutes at 37 °C in a shaking water bath. Cells were removed by centrifugation at 10000 g for 5 minutes at 4°C and ferricytochrome c in the supernatants was determined spectrophotometrically in duplicate samples at 550 nm using an extinction coefficient of 21 mM\(^{-1}\) cm\(^{-1}\) (Kuthan et al., 1982). The production of superoxide was inhibited > 99% by superoxide dismutase (500 U/ml). Superoxide formation in the absence of PMA was < 5% as compared to samples with PMA included. For inhibition studies, samples containing rat neutrophils were incubated in the presence of propylthiouracil (5-100 \(\mu\)M), methimazole (5-100 \(\mu\)M), diphenylene iodonium chloride (0.25-25 \(\mu\)M) or desferrioxamine (100 \(\mu\)M).

5.2.6 A1PI Activity

The elastase inhibitory activity of A1PI was measured as described previously (Travis and Johnson, 1982). Reaction mixtures contained neutrophils (1 x 10\(^6\) cells), A1PI (138 \(\mu\)g/ml), PMA (100 ng/ml) and HBSS (pH 7.4) in a total volume of 250 \(\mu\)l. Additional samples were prepared as described above, with the addition of propylthiouracil (100 \(\mu\)M), diphenylene iodonium chloride (10 \(\mu\)M) or desferrioxamine (100 \(\mu\)M). Samples were incubated for 30 minutes at 37 °C, placed on ice for 3 minutes and centrifuged at 14000 g for 5 minutes. Porcine pancreatic elastase (1.5
U/ml) was combined with 100 µl of the cell-free supernatant and 0.2 M Tris buffer (pH 8.0) in a total volume of 500 µl. After a 10 minute incubation at room temperature SLAPN (0.9 mM) was added and the increase in absorbance at 410 nm was measured spectrophotometrically in duplicate samples over a 3 minute interval. The rate of formation of p-nitroanilide was calculated using an extinction coefficient of 8.8 mM⁻¹ cm⁻¹ (Bieth et al., 1974). Results were expressed as a percentage in comparison to samples incubated with A1PI in the absence of neutrophils and inhibitors. In these samples elastase activity was inhibited by 50-75%. Inhibitors had no effect on the activity of A1PI in the absence of activated neutrophils and did not directly interfere with the assay of elastase activity.

5.2.7 Electron Spin Resonance (ESR)/Spin Trapping of α-hydroxyethyl Radicals

The ability of stimulated neutrophils to form hydroxyl radicals was measured using the spin trapping of the α-hydroxyethyl radical with 4-POBN and ESR detection of the stable 4-POBN:α-hydroxyethyl spin-trap adduct (Ramos et al., 1992). Rat or chicken neutrophils (2 x 10⁷ cells/ml) were combined with 4-POBN (20 mM), ethanol (170 mM), DTPA (100 µM), PMA (100 ng/ml) and HBSS (pH 7.4) in a total volume of 300 µl. Inhibition studies were carried out with the addition of propylthiouracil (100 µM), diphenylene iodonium chloride (10 µM) or desferrioxamine (100 µM). Samples were vortexed, immediately transferred to a quartz ESR flat cell cuvette and placed in the cavity of a Bruker Instruments ER200D/ESP 3220 spectrometer system. Spectra were recorded over 15 minutes at 37.0 ± 0.5°C with instrument settings as follows: microwave frequency 9.63 GHz; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.0 G; sweep width 65 G; sweep time 84 sec; number of scans 10 and time constant 82 msec.
5.2.8 Cytotoxicity

Neutrophil viability in the presence of test compounds was assessed by trypan blue exclusion. Rat neutrophils (5 x 10^6 cells/ml) were suspended in HBSS with PMA (100 ng/ml), propylthiouracil (100 μM), diphenylene iodonium chloride (10 μM) or desferrioxamine (100 μM) in a total volume of 500 μl and incubated for 30 minutes at 37 °C. An aliquot of the mixture was combined 1:1 with 0.4% trypan blue in 0.9% saline and the percentage of trypan blue-negative neutrophils was determined using a hemocytometer. The viability of rat neutrophils incubated with these agents did not differ significantly from that of control neutrophils. All viabilities exceeded 90%.

5.2.9 Statistics

Results are expressed as the mean ± standard error for a minimum of 3 experiments. Differences between groups were determined by analysis of variance followed by Newman-Keuls post-hoc tests. Results were considered significant at p < 0.05.

5.3 Results

Hypochlorous acid was generated by rat neutrophils (1 x 10^6 cells/ml) activated with PMA (100 ng/ml) at the rate of 16.1 ± 2.7 nmoles/10^6 cells/30 minutes, but was not formed by non-activated neutrophils. Propylthiouracil and methimazole inhibited the synthesis of hypochlorous acid by rat neutrophils (Fig. 15) with an IC_{50} of 6 μM for propylthiouracil and 20 μM for methimazole. The NADPH oxidase inhibitor diphenylene iodonium chloride also inhibited HOCl formation, with an IC_{50} of 0.2 μM.

Rat neutrophils stimulated with PMA generated superoxide at a rate of 23.9 ± 2.2 nmoles/10^6 cells/30 minutes. In the absence of PMA the synthesis of superoxide was negligible
Figure 15: Effect of inhibitors on hypochlorous acid formation by PMA-stimulated rat neutrophils. The generation of hypochlorous acid was measured by taurine trapping in cell-free supernatants following incubation for 30 minutes at 37°C. Reaction mixtures contained rat neutrophils (5 x 10^6/ml) suspended in HBSS with PMA (100 ng/ml), taurine (20 mM) (Control) and either 5-100 μM propylthiouracil (PTU), 5-100 μM methimazole (MMI) or 0.25-10 μM diphenylene iodonium chloride (DPI). Results are expressed as a percentage (mean ± SEM) compared to control (uninhibited) neutrophils (16.1 ± 2.7 nmoles/10^6 cells).
(<5 %) (not shown). The antithyroid agents propylthiouracil and methimazole (up to 100 μM studied) were without effect on superoxide synthesis (Fig. 16). The NADPH oxidase inhibitor diphenylene iodonium chloride (10 μM) completely inhibited the synthesis of superoxide (IC₅₀ = 0.20 μM).

Figure 17 shows the inhibitory effect of activated rat neutrophils on the activity of A1PI in the elastase assay. PMA-activated neutrophils reduced the inhibitory potency of A1PI to 35% of control values, thus markedly increasing elastase activity. Propylthiouracil (100 μM) inhibited the inactivation of A1PI exerted by stimulated neutrophils by about 90%, which was not significantly different from A1PI activity in the absence of activated neutrophils. The NADPH oxidase inhibitor diphenylene iodonium chloride (10 μM) blocked neutrophil-mediated A1PI inactivation by approximately 60%, while the iron chelator desferrioxamine (100 μM) had no effect on the neutrophil-induced inactivation of A1PI.

The generation of hydroxyl radicals by neutrophils was determined from the reaction of hydroxyl radical with ethanol. Stimulation of rat neutrophils by PMA in the presence of ethanol (170 mM), the iron chelator DTPA (100 μM) and the spin trap 4-POBN resulted in a 6-line ESR spectrum (Fig. 18), with hyperfine splitting constants characteristic of the α-hydroxyethyl adduct of 4-POBN (aN=15.6 G, aH=2.6 G) (Ramos et al., 1992). Neither propylthiouracil (100 μM) nor the iron chelating agent desferrioxamine (100 μM) affected the synthesis of hydroxyethyl radicals. In contrast, the NADPH oxidase inhibitor diphenylene iodonium chloride (10 μM) fully inhibited the hydroxyethyl radical-generated ESR signal. To confirm the identity of the 4-POBN spin trap adduct, [1,2-¹³C] ethanol was substituted for [¹³C] ethanol in the incubations. The resulting 12-line spectrum confirmed that the trapped species was in fact derived from ethanol.

Myeloperoxidase-deficient chicken neutrophils stimulated with PMA (100 ng/ml) generated superoxide at a rate of 30.0 ± 3.0 nmol/30 minutes/10⁶ cells, but did not generate
Figure 16: Effect of inhibitors on superoxide formation by PMA-stimulated rat neutrophils. Superoxide production was measured as the rate of superoxide dismutase-inhibitable cytochrome c reduction. Reaction mixtures contained rat neutrophils (2 x 10^6/ml) suspended in HBSS, PMA (100 ng/ml), 80 μM cytochrome c (Control) and either 5-100 μM propylthiouracil (PTU), 5-100 μM methimazole (MMI) or 0.25-10 μM diphenylene iodonium chloride (DPI). Results are expressed as a percentage (mean ± SEM) compared to control (uninhibited) neutrophils (23.9 ± 2.2 nmol/10^6 cells).
Figure 17: Effect of inhibitors on neutrophil-mediated A1PI inactivation. Baseline A1PI activity was determined from the inhibition of elastase activity in uninhibited cell-free samples containing 138 µg/ml A1PI (no cells). A1PI inactivation was determined in samples containing $1 \times 10^6$/ml rat neutrophils suspended in HBSS with 100 ng/ml PMA (PMN) and either 100 µM propylthiouracil (+ PTU), 10 µM diphenylene iodonium chloride (+ DPI) or, 100 µM desferrioxamine (+ DFO). Results are expressed as a percentage (mean ± SEM) of control (cell-free) samples. * indicates significant difference compared to cell-free samples as determined by ANOVA and post-hoc Newman-Keuls test.
Figure 18: ESR/4-POBN spin trapping of α-hydroxyethyl radicals in PMA-stimulated rat neutrophils. Incubations consisted of (a) $2 \times 10^7$/ml neutrophils in HBSS (pH 7.4) with 170 mM ethanol, 20 mM 4-POBN, 100 μM DTPA and 100 ng/ml PMA. B-F were prepared as in A and also contained the following (b) no PMA (c) 100 μM propylthiouracil (PTU) (d) 10 μM diphenylene iodonium chloride (DPI) (e) 100 μM desferrioxamine (DFO) (f) $^{13}$C ethanol.
hypochlorous acid (<0.8 nmoles/30 minutes/10⁸ cells). The generation of hydroxyl radicals by chicken neutrophils (Fig. 19) was of the same order of magnitude as that observed with rat neutrophils. Propylthiouracil (100 μM) did not affect the generation of hydroxyl radicals but diphenylene iodonium chloride (10 μM) fully inhibited the hydroxyethyl radical-generated ESR signal, indicating that in these cells, as in rat neutrophils, hydroxyl radical formation is completely dependent on NADPH oxidase activity but occurs independently of myeloperoxidase.

5.4 Discussion

This study offers several insights into the possible mechanism by which antithyroid drugs can protect against toxin-induced cell injury. In the present investigation, antithyroid drugs showed a concentration-dependent inhibition of myeloperoxidase-dependent hypochlorous acid formation in stimulated rat neutrophils. Hypochlorous acid formation was inhibited by propylthiouracil at concentrations (IC₅₀ = 6 μM) well below those used in the therapy of alcoholic liver disease (peak concentration of 43 μM following a 300 mg oral dose of PTU) (Orrego et al., 1987, Long et al., 1983).

Propylthiouracil has been reported to inhibit the catalytic activities of both myeloperoxidase (Taurog and Dorris, 1992) and thyroid peroxidase (EC 1.11.1.7) (Taurog and Dorris, 1989). The latter enzyme plays an important role in the synthesis of thyroid hormones (DeGroot and Niepomnischcze, 1977) and displays considerable amino acid homology with myeloperoxidase (Kimura and Ikeda-Saito, 1988). In early studies we postulated that drug-induced hypothyroidism was the main cause for the protection of alcohol induced liver injury (Israel et al., 1975). However, subsequent studies showed that in some forms of toxin-induced liver injury, such as that produced by galactosamine and acetaminophen, propylthiouracil exerts a protective effect independently of circulating thyroid hormone levels (Raheja et al., 1982; Cooper
Figure 19: ESR/4-POBN spin trapping of α-hydroxyethyl radicals in PMA-stimulated chicken neutrophils. Incubations consisted of (a) 2 x 10^7/ml cells in HBSS (pH 7.4) with 170 mM ethanol, 20 mM 4-POBN, 100 µM DTPA and 100 ng/ml PMA. B-F were prepared as in A and also contained the following (b) no PMA (c) 100 µM propylthiouracil (PTU) (d) 10 µM diphenylene iodonium chloride (DPI) or (e) 100 µM desferrioxamine (DFO).
These observations are in line with an inhibition by antithyroid drugs of myeloperoxidase rather than thyroid peroxidase contributing to the hepatoprotective effect. Inhibition of myeloperoxidase-dependent hypochlorous acid formation may be an important mechanism of hepatoprotection since it can promote tissue injury by (i) inactivating A1PI or (ii) generating hydroxyl radicals. A reduction in A1PI activity, resulting from either its inactivation or deficiency, has been implicated in a number of disease states such as emphysema, cystic fibrosis and rheumatoid joint disease in which oxidized A1PI and or elevated levels of free elastase are detectable in tissue fluids (Gadek and Crystal, 1983; Wong and Travis, 1980). In the present study propylthiouracil afforded nearly complete protection of A1PI against neutrophil-mediated damage, an effect which may account for the therapeutic effect of propylthiouracil in the treatment of alcoholic liver disease and other forms of toxin-induced liver injury. The possible involvement of neutrophil-mediated inactivation of A1PI as a mechanism in alcoholic liver injury has not yet been extensively investigated, but is suggested by the enhanced susceptibility to spontaneous and alcohol-induced liver disease in individuals with genetic A1PI deficiency. The incidence of the heterozygous A1PI variant in patients with alcoholic liver disease is reported to be 250% higher than in the healthy population, while homozygotes for the mutant A1PI allele frequently develop spontaneous liver fibrosis and cirrhosis (Pott et al., 1983). Acetaldehyde, generated during ethanol metabolism, can also inactivate A1PI and may help promote neutrophil-mediated proteolytic injury of the liver (Brecher et al., 1994).

The results of the present study indicate not only a mechanism by which propylthiouracil may protect the liver from toxin-induced injury, but also the possibility that propylthiouracil may be effective in treating a variety of other diseases characterized by neutrophil-mediated tissue injury. In support of this conclusion is the recent demonstration that psoriasis, an autoimmune
condition characterized by neutrophil accumulation, can also be effectively treated with propylthiouracil (Elias et al., 1994).

Since myeloperoxidase and hypochlorous acid have previously been shown to contribute to the formation of hydroxyl radicals by neutrophils (Ramos et al., 1992; Candieas et al., 1993), it was hypothesized that antithyroid drugs would inhibit hydroxyl radical formation and that this may be a potential mechanism of hepatoprotection. However, the data presented here showed that hydroxyl radical generation, measured by electron spin resonance as the hydroxyethyl radical, was not altered by propylthiouracil at concentrations sufficient to fully inhibit the myeloperoxidase-dependent production of hypochlorous acid. In addition, the lack of effect of propylthiouracil on hydroxyethyl radical formation tends to rule out the possibility that propylthiouracil acts as a hydroxyl radical scavenger in vivo, as has been suggested on the basis of prior studies using cell-free systems (Hicks et al., 1992). These observations prompted additional studies to delineate the enzymatic mechanisms of hydroxyl radical production in neutrophils.

5.4.1 A Role for Myeloperoxidase and Hypochlorous Acid in Hydroxyl Radical Generation?

The lack of involvement of myeloperoxidase and hypochlorous acid on the formation of hydroxyl radicals was suggested by the inability of propylthiouracil to inhibit hydroxyl radical generation. This was further confirmed by the finding that myeloperoxidase-null chicken neutrophils, which did not generate hypochlorous acid, produced hydroxyl radical at rates that were comparable to those observed in myeloperoxidase-rich rat neutrophils. In contrast, the production of superoxide anion was essential to generate hydroxyl radicals, as seen by the complete abolition of hydroxyethyl radical production in the presence of the NADPH oxidase inhibitor diphenylene iodonium chloride, in both chicken and rat neutrophils. In order to minimize the contribution of hydroxyl radical formation by the iron-dependent Haber-Weiss reaction, the
incubation buffers in the present study were treated with Chelex resin to remove free iron and contained the iron chelator DTPA. Hydroxyl radical formation was evident despite these procedures and further, was not affected by substitution of DTPA with the more potent iron chelator desferrioxamine. Prior studies suggest that in neutrophils hydroxyl radical production by the Haber-Weiss reaction, which requires free intracellular iron as a catalyst, occurs only when exogenous ferrous iron (Fe^{2+}) is added to the neutrophil incubation medium (Ramos et al., 1992). In our experience, addition of ferrous iron to a physiological buffer containing ethanol generated hydroxyethyl radicals \textit{per se} even in the absence of neutrophils (unpublished data). This observation confirms studies by Reinke et al. (1994b) showing that autooxidation of Fe^{2+} generates species that react with ethanol to produce hydroxyethyl radicals, without the involvement of a hydroxyl radical intermediate. The ability of neutrophils to endogenously generate hydroxyl radical \textit{via} the Haber-Weiss reaction has also been questioned on the basis that the availability of free iron in the neutrophil may be limited by the iron binding protein lactoferrin (Gutteridge et al., 1981). However, since the actual concentration of free iron in neutrophils is unknown and the ability of extracellular iron chelators to substantially alter intracellular free iron is not clear, hydroxyl radical synthesis in neutrophils by the Haber-Weiss reaction cannot be ruled out.

An alternative explanation for the ability of rat neutrophils to generate hydroxyl radical \textit{via} superoxide in the absence of exogenous iron, is that the hydroxyl radical is not derived from the Haber-Weiss reaction, but rather from the decomposition of peroxynitrite. Peroxynitrite is formed by the reaction of superoxide and nitric oxide radicals, and decomposes to produce hydroxyl radicals by a mechanism that is iron-independent (Beckman et al., 1990). A variety of cell types including neutrophils, which simultaneously generate nitric oxide and superoxide (Rodenas et al.,
1995), might in addition produce hydroxyl radicals from the decomposition of peroxynitrite. Future studies should determine the relative role of this mechanism.

In summary, the data obtained in the present studies indicate that inhibition of neutrophil-mediated hypochlorous acid formation and A1PI inactivation, rather than decreased hydroxyl radical formation or a hydroxyl radical scavenging effect, are the likely mechanisms by which antithyroid drugs protect against neutrophil-mediated tissue injury in a variety of pathological conditions.
6. GENERAL DISCUSSION

The studies in this thesis examined several potential mechanisms which may contribute to the ability of PTU to minimize ethanol- and other forms of toxin-induced liver injury. The focus of these experiments was on (a) the interaction of PTU with NADPH-cytochrome P450 reductase (CYP-reductase) and the ethanol-inducible P450 isozyme CYP2E1 and (b) the effect of PTU on circulating neutrophil counts and the ability of these cells to generate reactive oxygen species (ROS).

In the first study presented we investigated the effect of PTU on hepatic microsomal oxygen consumption, ROS formation and ethanol metabolism and on the activities of CYP-reductase and CYP2E1 in rats receiving ethanol or acetone chronically. In agreement with prior studies, chronic treatment with ethanol for 29 days resulted in significant induction of CYP2E1 (Lieber, 1988), as shown by increases in both CYP2E1 protein levels (300%) and metabolism of the CYP2E1 substrate NDMA (200%). Accompanying the induction of CYP2E1 in ethanol-treated rats was a substantial elevation in the rates of oxygen consumption (70%), hydroxyl radical (OH·) production (49%) and ethanol oxidation (50%). Chronic acetone treatment for 14 days produced a similar induction of CYP2E1-mediated NDMA metabolism (>200%) and ethanol oxidation (47%), but induced oxygen consumption to a lesser extent (35%) and did not affect the formation of OH·.

Increases in microsomal CYP-reductase activity have been reported previously in rats receiving chronic ethanol (Ekstrom and Ingelman-Sundberg, 1989; Joly et al., 1973; Lieber and DeCarli, 1970b; Shaw, 1989), however other studies have failed to confirm this finding (French et al., 1983a; Badger et al., 1993a). In the present study, chronic ethanol treatment resulted in overall increases in CYP-reductase protein levels and CYP-reductase activity of 15% and 46%, respectively, however the effect was highly variable between rats and was not statistically
CYP-reductase can contribute to microsomal oxygen consumption, \( \text{OH}^- \) formation and substrate oxidation by two mechanisms. First, CYP-reductase serves as a rate-limiting coenzyme in CYP2E1-mediated electron transfer reactions. Second, in the presence of iron, CYP-reductase can auto-oxidize thereby consuming oxygen and generating \( \text{OH}^- \) independently of CYP2E1. In the present study, CYP reductase induction, rather than an increase in CYP2E1 levels or activity, would account for the increased production of \( \text{OH}^- \) observed in microsomes from ethanol-treated rats, since chronic acetone treatment produced an equal degree of induction of CYP2E1 activity but did not affect CYP-reductase activity or \( \text{OH}^- \) formation. Similarly, although both acetone and ethanol treatments enhanced the rate of microsomal oxygen consumption, the increase in ethanol-treated rats (70%) was considerably more pronounced than in the acetone-treated rats (35%), suggesting that CYP-reductase activity also contributes to microsomal oxygen consumption. Together these findings indicate that increases in both CYP2E1 and CYP-reductase in liver microsomes can result from chronic ethanol feeding, and that both effects contribute to increased rates of microsomal oxygen consumption and \( \text{OH}^- \) formation.

Administration of PTU (25 mg/kg/day\(^8\)) reduced serum \( T_3 \) and \( T_4 \) levels by 64% in ethanol-, acetone-treated and control rats and also lowered the activity and protein levels of CYP-reductase by 27-34\% and 66-69\%, respectively. This observation is consistent with previous reports that reduction of thyroid hormone levels by administration of antithyroid drugs such as PTU (Raheja et al., 1985) or methimazole (Ram and Waxman, 1992), or by surgical manipulations (Ram and Waxman, 1992; Rumbaugh et al., 1978; Waxman et al., 1989), lowers microsomal CYP-reductase levels and/or activity. Associated with the lowered levels of CYP-reductase in PTU-treated rats was a decrease in the rates of microsomal oxygen consumption.

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\(^8\) This dose of PTU was shown to lower circulating thyroid hormone levels and hepatic microsomal CYP-reductase activity (see Appendix 2).
OH- production and ethanol oxidation; effects which are likely to contribute to the hepatoprotective properties of PTU reported previously.

This study is the first to report that PTU attenuates the increase in hepatic microsomal oxygen utilization induced by chronic ethanol consumption. This observation is consistent with the findings that PTU administration in chronic ethanol-fed rats reduces hepatic oxygen utilization both in isolated perfused livers (Rachamin et al., 1985; Thurman et al., 1984a; 1984b), and in the intact animal (Carmichael et al., 1993), and attenuates hypoxia-induced liver injury (Israel et al., 1975; Britton et al., 1979) and provides a plausible biochemical mechanism to account for these effects. The induction of CYP2E1 in ethanol-treated rats occurs preferentially in zone III of the liver (Buhler et al., 1991; Ingelman-Sundberg et al., 1988) where circulating oxygen concentrations are the lowest and which is particularly susceptible to ethanol-induced damage (French et al., 1993). CYP2E1 induction can contribute significantly to hepatic oxygen consumption (Thurman et al., 1984a; 1984b; Tindberg and Ingelman-Sundberg, 1989) and may promote hypoxic injury to centrilobular hepatocytes. Thus, the effect of PTU on microsomal oxygen consumption observed in vitro could account for the ability of PTU to counteract the ethanol-induced hypermetabolic state and hypoxia-induced liver injury in vivo.

The lowered levels of CYP-reductase in PTU-treated rats was also associated with a decrease in the rate of microsomal OH- formation. The ability of PTU to attenuate the ethanol-induced enhancement of microsomal OH- formation, via decreased levels of CYP-reductase, represents another possible mechanism to explain the drug’s hepatoprotective properties. Enhanced production of OH- associated with chronic ethanol ingestion may contribute to a variety of potentially damaging free radical reactions with lipids, proteins and DNA that may play an important role in promoting liver injury. Compounds that limit the potential for these reactions to occur, such as OH- scavengers, have been shown to protect against tissue injury in vitro and in vivo.
vivo and have received considerable interest as potential therapeutic agents (Ward et al., 1983; Fox, 1984; Vercelotti et al., 1985; Weitberg et al., 1985).

An alternate explanation for the reduction in OH- formation in PTU-treated rats stems from prior findings that PTU significantly decreases hepatic iron content in rats (Al-Khayat et al., 1982) and prevents the increase in liver iron content associated with chronic ethanol treatment in mice (Gonzalez-Reimers et al., 1989). Iron serves as a catalyst for the formation of OH- by the Haber-Weiss reaction and it is possible that OH- formation, and the liver injury that may result, could be partially dependent on hepatic iron concentration. Prior studies have shown that hepatic non-heme iron content may be elevated in rats receiving chronic ethanol (Shaw 1989) and in human alcoholics with ALD (Williams et al., 1967; Zimmerman et al., 1961) and that increased hepatic iron content is associated with more severe liver injury in rats receiving chronic ethanol treatment (Stal and Hultcrantz, 1993; Tsukamoto et al., 1992). However, studies presented in this thesis (Appendix 3), indicate that hepatic non-heme iron content is not significantly altered in either ethanol- or PTU-treated rats. The discrepancy between the results obtained and those reported in previous investigations on the effect of PTU on hepatic iron levels (Al-Khayat et al., 1982; Gonzalez-Reimers et al., 1989) are likely due to differences in the dose and method of administration, however this cannot be confirmed as the exact dose of PTU used in those studies was not reported. Since PTU did not alter the hepatic concentration of non-heme iron in rats, the observation that PTU reduces microsomal OH- formation appears to be solely attributable to the lower levels of CYP-reductase, rather than decreased availability of catalytic iron in the liver.

It was also shown that PTU reduced the microsomal oxidation of ethanol to acetaldehyde (MEOS activity) but did not affect the metabolism of other CYP2E1 substrates such as NDMA. This finding is in line with other studies which indicate that OH- generated from the NADPH-dependent autoxidation of CYP-reductase contributes to ethanol oxidation by liver microsomes in
vitro, independently of CYP2E1 (Cederbaum, 1989; Winston and Cederbaum, 1983b). Thus, the reduction in MEOS activity observed in microsomes from PTU-treated rats can be attributed to the decrease in OH- generation associated with lowered levels of CYP-reductase. Acetaldehyde is a highly toxic compound which has been postulated to play an important role in the pathogenesis of alcohol-induced liver injury. Therefore, the ability of PTU to inhibit the microsomal oxidation of ethanol and generation of acetaldehyde represents an additional mechanism that may contribute to the hepatoprotective effect.

In summary, chronic ethanol ingestion results in the induction of CYP2E1 and, to a lesser extent CYP-reductase and enhances microsomal oxygen utilization, OH- production and acetaldehyde formation; processes which may be involved in promoting ethanol-induced liver pathologies. The ability of PTU to minimize these effects by lowering CYP-reductase levels may contribute to its therapeutic efficacy in preventing toxin-induced liver injury.

The finding that PTU counteracts the potentially damaging effects of CYP2E1 induction on the liver may also explain why liver injury caused by carbon tetrachloride (Orrego et al., 1987) and acetaminophen (Yamada et al., 1981; Linscheer et al., 1980; Raheja et al., 1982), which are known substrates of CYP2E1, is prevented by PTU administration.

The ability of PTU to modulate CYP-reductase by lowering thyroid hormone levels not only provides a plausible mechanism for the hepatoprotective effects of PTU, but also explains the relationship between circulating thyroid hormone levels and liver injury previously demonstrated in several animal models. Surgical procedures leading to a reduction of thyroid hormone levels (i.e., hypophysectomy), which have been shown to lower CYP-reductase levels in liver microsomes (Ram and Waxman, 1992; Waxman et al., 1989), provide a similar degree of protection as PTU against hypoxia-induced liver injury in ethanol-fed rats (Israel et al., 1975b) and thioacetamide hepatotoxicity (Oren et al., 1996). Conversely, administration of T₄, (Waxman
et al., 1989), which has been shown to increase CYP-reductase levels in the liver (Ram and Waxman, 1992), potentiates liver injury induced by lindane (Videla et al., 1995), 1,1-dichloroethylene (Kanz et al., 1988), thioacetamide (Oren et al., 1996), volatile anesthetics such as halothane, isoflurane and enflurane (Berman et al., 1983; Uetrecht et al., 1983) and ischemia-reperfusion (Troncoso et al., 1997). The relationship between thyroid hormones, CYP-reductase levels and liver injury observed in these experiments is consistent with a thyroid hormone-mediated modulation of CYP-reductase in the hepatoprotective action of PTU. However, PTU also protects against other forms of tissue injury by mechanisms that are clearly independent of the drug’s effect on thyroid hormone and CYP-reductase levels. For example, PTU prevents acetaminophen-induced liver injury even when rats are made hyperthyroid by administration of T3 (Raheja et al., 1982). Similarly, galactosamine-induced hepatotoxicity is prevented by PTU in both hypothyroid and euthyroid rats and at doses which do not produce hypothyroidism (Cooper et al., 1984). Furthermore, galactosamine is not a known substrate of CYP2E1 and therefore the ability of PTU to prevent liver injury induced by this agent is not likely to be mediated by CYP-reductase. Finally, PTU has been shown to minimize skin cell injury associated with psoriasis (Elias et al., 1994), a condition in which there is no prior evidence to suggest the involvement of CYP2E1 and CYP-reductase. These observations clearly indicate that the thyroid hormone-mediated effect of PTU on CYP-reductase cannot be the only mechanism contributing to the drug’s general cytoprotective action.

An alternative mechanism investigated in the present studies is that the hepatoprotective action of PTU may be related to a reduction in the number of circulating neutrophils. A common feature in alcoholic liver disease (ALD) and other forms of tissue injury against which PTU has been shown to protect, is the presence of neutrophil infiltrates in inflamed tissues and increased numbers of neutrophils in the circulation (neutrophil leukocytosis). Reactive oxygen species and
proteolytic enzymes are released by activated neutrophils during the respiratory burst, both of which have been proposed to play an important role in mediating the cell injury associated with inflammation (Weiss, 1989; Smith, 1994). PTU is oxidized by activated neutrophils to form electrophillic metabolites that may be directly toxic to circulating neutrophils or their bone marrow precursors, or may haptenize neutrophil components and trigger an autoimmune response (i.e., anti-neutrophil antibodies) that results in the destruction of circulating neutrophils. This mechanism has been proposed (Waldhauser and Utrecht, 1991) to explain the reduction in circulating neutrophils (i.e., neutropenia and agranulocytosis) which is occasionally observed in patients receiving PTU for the treatment of Grave’s disease (Wing and Aspen, 1952; Amrheim et al., 1970; Kariya et al., 1983; 1984; Trotter, 1962; Rosove, 1977; Cooper et al., 1983). In theory, reductions in circulating neutrophil counts induced by PTU could counteract the increase in circulating neutrophils and the subsequent infiltration of neutrophils in the liver associated with ALD, thus constituting a potentially important mechanism contributing to the drug’s hepatoprotective effects.

In the present investigations, the effect of PTU administration on circulating neutrophil counts was examined in rats receiving intragastric infusion of either ethanol-containing or control liquid diets. Circulating neutrophil counts were not significantly affected in either control or ethanol-treated rats following 4 days of treatment with PTU at doses in the range of 1-50 mg/kg/day. The lack of effect of PTU on circulating neutrophil counts observed in the present study, differs from the results previously reported by Kariya et al. (1983; 1984), however the dose of PTU used (255 mg/kg/day i.p.) was considerably higher than that used in the present study (1-55 mg/kg/day p.o.) and greatly exceeds the doses typically used in the treatment of ALD (Orrego et al., 1987). Based on the failure of PTU to induce neutropenia at therapeutically relevant doses in both control and ethanol-treated rats, it can be concluded that this is not a mechanism
contributing to the hepatoprotective effect of PTU against ethanol-induced liver injury.

Studies were also conducted to determine the relationship between circulating neutrophils and liver injury in two widely used rat models of experimental ALD - the oral diet feeding method of Lieber and DeCarli (1982) and the continuous intragastric infusion method of Tsukamoto and French (Tsukamoto et al., 1985). Liver injury was observed in ethanol-treated rats using both techniques, however as predicted, the extent of liver damage observed was considerably greater using the intragastric infusion method of ethanol administration. Furthermore, the liver injury induced with this technique was accompanied by a significant increase in circulating neutrophil counts, which was not observed using the oral diet feeding method. This finding, together with the observation that neutrophil leukocytosis is commonly observed in humans with ALD, suggested that increases in circulating neutrophils may somehow contribute to alcohol-induced liver injury. However, several additional observations tended to discount this possibility. First, although circulating neutrophil counts were elevated in rats receiving ethanol by the intragastric infusion method, widespread infiltration of neutrophils in the liver, a condition which would be necessary in order for neutrophil-mediated tissue damage to occur, was not observed. Secondly, administration of G-CSF in rats receiving ethanol by the oral feeding technique produced a 6-9 fold increase in circulating neutrophil counts, but did not potentiate liver injury. Several investigators have observed alcohol-induced hepatitis and hepatic neutrophil infiltration using the intragastric ethanol infusion technique, however a longer duration of ethanol treatment (> 3 months) than that used in the present study may be necessary in order for this to occur (Tsukamoto et al., 1992; 1990). The use of a longer-term model of continuous intragastric ethanol infusion may ultimately be better suited to study the role of neutrophils in ethanol-induced liver injury, however due to the considerable time, labor and cost requirements, such a study was well beyond the scope of the present investigations.
In summary, our findings indicate that (i) elevations in circulating neutrophils do not directly contribute to ethanol-induced liver injury in either the oral diet feeding model or the continuous intragastric infusion model, and (ii) that the mechanism by which PTU protects against ethanol-induced liver injury does not involve a reduction in circulating neutrophils.

The final series of experiments presented in this thesis examined the interaction of PTU with the neutrophil enzyme myeloperoxidase (MPO) as a potential mechanism of hepatoprotection. MPO catalyzes the formation of reactive oxygen species (i.e., HOCl and OH\textsuperscript{-}) which have been strongly implicated as mediators of tissue injury during inflammation (Weiss, 1989; Smith, 1994).

In the present studies, antithyroid drugs (PTU and methimazole) produced a dose-dependent inhibition of MPO-dependent HOCl formation in rat neutrophils in vitro. The concentration of PTU required to inhibit HOCl formation in isolated neutrophils (IC\textsubscript{50} = 6 μM) is comparable to that reported previously in studies using purified MPO (Lee et al., 1990). PTU is administered at doses of 300 mg per day in the therapy of ALD (Orrego et al., 1987), which results in plasma PTU concentrations of approximately 43 μM (Long et al., 1983), while doses as high as 1000 mg per day may be used in the treatment of Grave’s disease (Cooper, 1984). The present results therefore indicate that full inhibition of MPO activity and HOCl formation would occur at PTU concentrations achieved during the therapy of ALD and well below the concentrations reached during the therapy of hyperthyroidism. Inhibition of MPO-dependent HOCl formation may therefore be an important mechanism of hepatoprotection, since HOCl can promote tissue injury by (i) the direct action of HOCl and its chloramine derivatives on inflamed tissues, (ii) inactivating A1PI or (ii) generating OH\textsuperscript{-}.

A1PI is a powerful antiproteinase that inhibits the activity of neutrophil-derived proteolytic enzymes and thereby limits their potential to induce injury to host tissues.
Hypochlorous acid generated by neutrophils can inactivate AIPI, resulting in enhanced susceptibility of host cells to proteolytic damage. A reduction in AIPI activity, resulting from either its inactivation or deficiency, has been implicated in a number of disease states such as emphysema, cystic fibrosis and rheumatoid joint disease in which oxidized AIPI and or elevated levels of free elastase are detectable in tissue fluids (Laurell and Eriksson 1963; Gadek and Crystal, 1983; Wong and Travis, 1980). In the present study PTU afforded nearly complete protection of AIPI against neutrophil-mediated damage, an effect which may account for the therapeutic effect of PTU in the treatment of ALD and other forms of toxin-induced liver injury. Although the involvement of neutrophil-mediated AIPI inactivation in the pathogenesis of ALD has not been extensively investigated, such an association is suggested by: (i) the enhanced susceptibility to spontaneous and alcohol-induced liver disease in individuals with genetic AIPI deficiency (Pott et al., 1983); and (ii) the observation that acetaldehyde, a byproduct of ethanol metabolism, inactivates AIPI and may therefore help promote neutrophil-mediated proteolytic injury of the liver (Brecher et al., 1994; Brecher and Pavlock, 1992).

The possibility that inhibition of MPO activity is an underlying mechanism of PTU cytoprotection would account for several prior observations that could not be readily explained by previously proposed mechanisms. First, is the observation that in some forms of toxin-induced liver injury, such as that produced by galactosamine and acetaminophen, PTU exerts a protective effect independently of circulating thyroid hormone levels (Raheja et al., 1982; Cooper et al., 1984). Since antithyroid activity is not a requirement per se for MPO inhibition by PTU, but rather can be regarded as a side effect arising from similarities in the polypeptide sequences of MPO and the thyroid hormone-synthesizing enzyme thyroid peroxidase (Kimura and Ikeda-Saito, 1988), the ability of PTU to prevent these forms of toxin-induced liver injury independently of thyroid hormones is consistent with a MPO-dependent, thyroid hormone-independent mechanism.
of hepatoprotection. Secondly, this mechanism would also account for the effectiveness of PTU in preventing liver injury induced by galactosamine (Cooper et al., 1984) and in treating non-hepatic tissue injury such as that associated with psoriasis, an autoimmune disorder of the skin (Elias et al., 1994). The pathogenesis of these diseases is not known to involve CYP2E1, CYP-reductase or thyroid hormones, but both are characterized by the infiltration of neutrophils in injured tissues (Al-Tuwaijri et al. 1981; Braun-Falco and Maciejewski, 1977).

In addition to revealing the probable mechanisms of PTU hepatoprotection, these studies also offered insight into the mechanism by which neutrophils generate OH·. Since MPO and HOCl have previously been shown to contribute to the formation of OH· by neutrophils (Ramos et al., 1992; Candieas et al., 1993), it was predicted that antithyroid drugs would inhibit OH· formation and that this may be a potential mechanism of hepatoprotection. However, the data obtained showed that OH· generation, measured by electron spin resonance as the hydroxyethyl radical, was not altered by PTU at concentrations sufficient to fully inhibit the MPO-dependent production of HOCl. The lack of involvement of MPO and HOCl on the formation of OH· was further suggested by the finding that MPO-null chicken neutrophils, which did not generate HOCl, produced OH· at rates that were comparable to those observed in MPO-rich rat neutrophils. Conversely, OH· formation was shown to be dependent on the production of O2· (i.e., NADPH oxidase activity), since the NADPH oxidase inhibitor diphenylene iodonium chloride completely prevented neutrophil hydroxyethyl radical production. These results suggested that the formation of OH· that was observed, occurred via the iron-catalyzed Haber-Weiss reaction. However, OH· formation was observed despite the pretreatment of buffers with chelating resin to minimize trace iron contamination and the inclusion of the iron chelating agents DTPA or DFO. Two possible explanations were given to account for this observation. First, is that these procedures may not be fully effective in minimizing intracellular free iron concentrations, and thus sufficient iron may
remain within the neutrophil to catalyze OH· synthesis by the Haber-Weiss reaction. A second possibility that would explain the formation of OH· in the absence of catalytically active iron is that the OH· is not derived from the Haber-Weiss reaction, but rather from the decomposition of peroxynitrite (ONOO−). Peroxynitrite is formed by the reaction of O2− and nitric oxide radicals (NO·), and decomposes to produce OH· by a mechanism that is iron-independent (Beckman et al., 1990). A variety of cell types including neutrophils, which simultaneously generate NO· and O2− (Rodenas et al., 1995; 1996), might in addition produce OH· from the decomposition of ONOO−. The contribution of this pathway to the generation of OH· by neutrophils and its role in neutrophil-mediated tissue is an area that warrants further investigation.

In summary, the studies presented in this thesis demonstrate two mechanisms that are likely to account for the cytoprotective properties of PTU. Firstly, PTU causes a thyroid hormone-dependent decrease in the level of CYP-reductase in liver microsomes. This offsets the induction of CYP2E1 and/or CYP-reductase, which can occur upon exposure to ethanol and other hepatotoxins, and therefore prevents increases in microsomal oxygen consumption, free radical generation and substrate toxification that may otherwise promote liver injury. Secondly, by mechanisms that are independent of its effects on thyroid hormone levels, PTU inhibits MPO activity in neutrophils thereby preventing HOCl formation and the proteolytic injury that may be induced by HOCl-mediated inactivation of A1PL. A combination of these two mechanisms can explain the cytoprotective effect of PTU on a diverse range of hepatic and non-hepatic inflammatory diseases which could not be readily accounted for by previously postulated mechanisms of action. Since the lack of a well defined mechanism of cytoprotection has limited the use of PTU therapy in treating such disorders, it is hoped that the results of these studies will rekindle interest in the potential benefits of PTU, and prompt additional clinical trials to evaluate the efficacy of PTU therapy in the treatment of tissue injury.
7. DIRECTIONS FOR FURTHER RESEARCH

7.1 Clinical Trials of Propyliouracil for the Treatment of Alcoholic Liver Disease (ALD)

Uncertainty regarding the mechanisms by which PTU protects against ethanol-induced liver injury has limited its clinical use in the treatment of ALD. The identification of the probable cytoprotective mechanisms of PTU in the present study provides a clear rationale for its use in ALD therapy and warrants that further clinical trials be undertaken to re-evaluate the efficacy of PTU therapy in the treatment of ALD. In order to provide conclusive results, such studies must include a sufficient number of patients to reliably avoid the possibility of a type II error and to control for high patient dropout rates. The failure to control for these factors has served as the basis for criticism of previous clinical trials with PTU in the therapy of ALD (Bloor et al., 1994). Other requisite design features include the use of placebo-control groups and the random assignment of patients to treatment groups. The use of heterogeneous patient populations has also been implicated as a potentially confounding factor in prior studies (Orrego et al., 1994), therefore the initial focus of the proposed clinical trials may be directed towards the patient subpopulation which was previously shown to have the most positive response to PTU - *i.e.*, abstinent patients and those without cirrhosis (Orrego et al., 1987; 1979; 1994). In order to provide sufficient numbers of such patients, the proposed studies would likely require the cooperative involvement of several treatment centres. Should the results of these studies show a positive effect of PTU therapy, a further objective will be to heighten awareness among clinicians of PTU as a therapeutic option for treating ALD.
7.2 Propylthiouracil Therapy in the Treatment of Other Forms of Tissue Injury

The present studies have identified two mechanisms - lowered levels of cytochrome P450-reductase in liver microsomes and inhibition of neutrophil myeloperoxidase - that are likely to contribute to the efficacy of PTU in the therapy of ALD. Since the mechanisms involved in the pathogenesis of ALD may be similar to those causing several other forms of inflammatory tissue injury, it is likely that PTU could also be effective in treating such conditions.

The reduction in CYP-reductase levels and activity resulting from PTU treatment was shown to counteract the increases in free radical generation, oxygen consumption and the formation of some reactive metabolites caused by treatment with CYP2E1-inducing agents (i.e., ethanol or acetone. Liver injury and other potentially damaging effects associated with CYP2E1 induction may also be produced by a variety of other CYP2E1 substrates to which humans are frequently exposed. A potential area for further research is to examine the possible clinical use of PTU for the treatment or prevention of liver injury induced by CYP2E1 substrates. Table 1 lists a variety of compounds which could be considered for such studies.

The present studies indicate that the PTU-mediated decrease in cytochrome P50-reductase was associated with a reduction in hepatic microsomal oxygen consumption and free radical formation. Another important observation was that these effects were also observed in control rats which did not receive treatments to induce CYP2E1 activity (i.e., administration of ethanol or acetone). Thus, in addition to exerting a specific protective effect against liver injury induced by CYP2E1 substrates, the ability of PTU to minimize oxygen consumption and free radical formation in control rats represents a more generalized effect that may be therapeutically beneficial in treating many other forms of liver disease. For example, PTU could conceivably be a useful adjunct in treating liver injury associated with ischemia-reperfusion or liver transplantation, conditions in which oxygen depletion (Iritia et al., 1996; Steltzer et al., 1993; Decker et al., 1993)
and/or free radical formation (Connor et al., 1994; Goode et al., 1994; Bzeizi et al., 1997; Marubayashi and Dohi, 1996) may contribute to liver damage, independently of CYP2E1.

The present studies have also shown that PTU inhibits the generation of hypochlorous acid by the neutrophil enzyme myeloperoxidase, and prevents neutrophil-mediated inactivation of A1PI. This action minimizes the tissue-destructive potential of neutrophils and is likely to contribute to the efficacy of PTU in the treatment of ALD. Neutrophil infiltration in damaged tissues is a characteristic feature of ALD and many other forms of inflammatory tissue injury. Oxidative attack by neutrophil-derived reactive oxygen species, and the inactivity of A1PI - resulting in enhanced susceptibility of host tissues to injury by neutrophil-derived proteolytic enzymes) - are processes which have been implicated in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (Pillinger and Abramson, 1995), emphysema (Rahman and MacNee, 1996; Doring, 1994; Evans and Pryor, 1994) and periodontitis (Travis et al., 1994) and in ischemia-reperfusion and organ transplantation injury (Korthuis, 1994). Our results suggest that PTU may be beneficial in the treatment of these and other neutrophil-related disorders. Prior studies demonstrating the effectiveness of PTU in preventing liver injury induced by galactosamine (Cooper et al., 1984) and in treating psoriasis (Elias et al., 1994), conditions characterized by the infiltration of neutrophils in injured tissues (Al-Tuwaijri et al. 1981; Braun-Falco and Maciejewski, 1977), further supports such a conclusion. Future studies, both at the experimental and clinical level, should therefore aim to evaluate the effectiveness of PTU in the treatment of various inflammatory diseases, particularly those of the liver, lung, skin and joints.

7.3 Propylthiouracil as a Prototype for the Development of Novel Therapeutic Agents

The present studies indicate that inhibition of MPO is likely to be one of the mechanisms by which PTU protects against several forms of tissue damage. The identification of this
mechanism provides a strategy for the design of novel pharmacological agents with increased potency and/or specificity, for the treatment of alcohol-induced and other forms of tissue injury. For example, the chemical structure of PTU can be modified to generate PTU analogs which may potentially have reduced systemic toxicity, but retain the anti-inflammatory properties of the parent molecule. The advantages and shortcomings of such an approach are discussed in greater detail in Appendix 4. An alternative to using PTU and related analogs to modulate MPO activity is to inhibit the production of the protein altogether by using gene-targeted oligonucleotide drugs. Antisense molecules are synthetic deoxyribonucleotides which contain nucleotide sequences complementary to those of an intended target mRNA (e.g. that coding for MPO). Antisense drugs bind specifically to the targeted mRNA and either promotes its degradation by RNAase H or blocks its translation, leading to a reduction in the levels of the encoded protein. Antisense therapies offer several potential advantages over conventional pharmacological agents as a means of inhibiting MPO activity, since in theory, they affect only a single mRNA population and are therefore exquisitely specific and lack the systemic toxicity of conventional therapeutic drugs. The potential application of antisense strategies in the development of novel MPO inhibitors is discussed in detail in Appendix 4.

7.4 Role of Peroxynitrite in the Generation of Hydroxyl Radicals by Neutrophils

The characterization of the mechanisms leading to hydroxyl radical formation by neutrophils is necessary in order to understand the pathology of inflammatory diseases and to design effective pharmacological treatments. The present studies suggested that the generation of OH· by neutrophils occurs independently of MPO activity, while being fully dependent on superoxide production and NADPH oxidase activity. In addition, iron chelators did not affect neutrophil OH· formation, suggesting that pathways other than the iron-catalyzed Haber-Weiss
reaction may be responsible for the production of OH\(^{-}\). An interesting possibility raised by these findings is that OH\(^{-}\) may be produced in neutrophils by the decomposition of peroxynitrite (ONOO\(^{-}\)). Peroxynitrite is formed by the reaction of O\(_2\)^{-}\(\cdot\) and nitric oxide radicals (NO\(^{\cdot}\)), and decomposes to produce OH\(^{-}\) by a mechanism that is iron-independent (Beckman et al., 1990). Since neutrophils can simultaneously generate nitric oxide and O\(_2\)^{-}\(\cdot\) (Rodenas et al., 1995; 1996), the generation of OH\(^{-}\) from the decomposition of peroxynitrite is also possible. This hypothesis could be tested in future studies by measuring OH\(^{-}\) formation in the presence of agents which inhibit nitric oxide biosynthesis, such as the arginine analogs N-omega-nitro-L-arginine methyl ester and L-NG monomethyl arginine (Moore and Handy, 1997; Pfeilschifter et al., 1996).
8. REFERENCES


175


9. APPENDIX 1

9.1 Recipes For Liquid Diets In Ethanol Administration Studies

Listed below are the various recipes used in the formulation of the ethanol-containing and isocaloric control liquid diets described in Studies 1 and 2. All recipes yield 1 L total volume of liquid diet with caloric content of 1.04 kcal/ml.

9.1.1 Study 1 - Control Diet for Oral Administration

<table>
<thead>
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<th>Amount</th>
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<th>% Calories</th>
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<td>viscarin</td>
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<td>-</td>
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<tr>
<td>maltose-dextrin</td>
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<tr>
<td>water</td>
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9.1.2 Study 1 - Ethanol Diet for Oral Administration

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<th>% Calories</th>
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<td>viscarin</td>
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<td>-</td>
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<tr>
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* Dry Mix Composition

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<td>Salt mix</td>
<td>10.0 g</td>
<td></td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>1.5 g</td>
<td></td>
</tr>
<tr>
<td>DL-methionine</td>
<td>269.0 mg</td>
<td></td>
</tr>
<tr>
<td>choline chloride</td>
<td>228.0 mg</td>
<td></td>
</tr>
</tbody>
</table>

* Oil mix consists of 80% corn oil, 12% olive oil and 8% cod liver oil.

187
### Study 1 - Oral Liquid Diets: Vitamin and Mineral Contents

**Vitamins:** Supplied as 1.505 g Bio-Serv vitamin mix #20315 per L of diet.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>1354.0</td>
<td>IU</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>152.0</td>
<td>IU</td>
</tr>
<tr>
<td>(\alpha)-tocopherol</td>
<td>7.5</td>
<td>mg</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>67.7</td>
<td>mg</td>
</tr>
<tr>
<td>inositol</td>
<td>7.5</td>
<td>mg</td>
</tr>
<tr>
<td>choline chloride</td>
<td>340.9</td>
<td>mg</td>
</tr>
<tr>
<td>menadione</td>
<td>3.4</td>
<td>mg</td>
</tr>
<tr>
<td>PABA</td>
<td>7.5</td>
<td>mg</td>
</tr>
<tr>
<td>niacin</td>
<td>6.8</td>
<td>mg</td>
</tr>
<tr>
<td>riboflavin</td>
<td>1.5</td>
<td>mg</td>
</tr>
<tr>
<td>pyridoxine HCl</td>
<td>1.5</td>
<td>mg</td>
</tr>
<tr>
<td>thiamine HCl</td>
<td>1.5</td>
<td>mg</td>
</tr>
<tr>
<td>calcium pantothenate</td>
<td>4.5</td>
<td>mg</td>
</tr>
<tr>
<td>biotin</td>
<td>30.0</td>
<td>(\mu)g</td>
</tr>
<tr>
<td>folic acid</td>
<td>140.0</td>
<td>(\mu)g</td>
</tr>
<tr>
<td>vitamin B(_{12})</td>
<td>2.0</td>
<td>(\mu)g</td>
</tr>
</tbody>
</table>

**Minerals:** Supplied as 10.03 g Bio-Serv salt mix #711-SY per L of diet.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Amount</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium</td>
<td>1.7</td>
<td>g</td>
</tr>
<tr>
<td>phosphorous</td>
<td>1.2</td>
<td>g</td>
</tr>
<tr>
<td>potassium</td>
<td>1.9</td>
<td>g</td>
</tr>
<tr>
<td>magnesium</td>
<td>213.0</td>
<td>g</td>
</tr>
<tr>
<td>sodium</td>
<td>665.0</td>
<td>mg</td>
</tr>
<tr>
<td>chloride</td>
<td>700.0</td>
<td>mg</td>
</tr>
<tr>
<td>iron</td>
<td>77.0</td>
<td>mg</td>
</tr>
<tr>
<td>sulfur</td>
<td>1.2</td>
<td>mg</td>
</tr>
<tr>
<td>manganese</td>
<td>935.0</td>
<td>(\mu)g</td>
</tr>
<tr>
<td>copper</td>
<td>689.7</td>
<td>(\mu)g</td>
</tr>
<tr>
<td>iodine</td>
<td>4.8</td>
<td>mg</td>
</tr>
<tr>
<td>zinc</td>
<td>28.0</td>
<td>mg</td>
</tr>
</tbody>
</table>

---

112.9 mg choline chloride from vitamin mix supplemented with 228.0 mg additional, for a total of 340.9 mg.
9.1.4 Study 2 - Oral Liquid Diets

The contents of the control and ethanol diets used in Study 2 were identical to those described above for Study 1, except that 4.0 g of vitamin mix was used instead of 1.505 g as indicated in the original recipe. The vitamin composition of the modified diets is listed below.

**Vitamins:** Supplied as 4.0 g Bio-Serv vitamin mix #20315.

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>3600.0 IU</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>400.0 IU</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>180.0 mg</td>
</tr>
<tr>
<td>i-inositol</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>choline chloride</td>
<td>300.0 mg</td>
</tr>
<tr>
<td>menadione</td>
<td>9.0 mg</td>
</tr>
<tr>
<td>PABA</td>
<td>20.0 mg</td>
</tr>
<tr>
<td>niacin</td>
<td>18.0 mg</td>
</tr>
<tr>
<td>riboflavin</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>pyridoxine HCl</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>thiamine HCl</td>
<td>4.0 mg</td>
</tr>
<tr>
<td>calcium pantothenate</td>
<td>12.0 mg</td>
</tr>
<tr>
<td>biotin</td>
<td>80.0 µg</td>
</tr>
<tr>
<td>folic acid</td>
<td>360.0 µg</td>
</tr>
<tr>
<td>vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>5.4 µg</td>
</tr>
</tbody>
</table>
9.1.5 Study 2 - Liquid Diets for Intragastric Infusion

The vitamin content of liquid diets used in intragastric infusion studies was identical to that described for the oral diet used in Study 2. Diet contents that differ from the unmodified diet are outlined below.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactalbumin hydrolysate</td>
<td>92.0 g/L</td>
</tr>
<tr>
<td>Dextrose</td>
<td>132.4 g/L</td>
</tr>
<tr>
<td>Corn oil</td>
<td>32.9 g/L</td>
</tr>
<tr>
<td>Citric acid</td>
<td>2.0 g/L</td>
</tr>
<tr>
<td>Tween-80</td>
<td>5.4 g/L</td>
</tr>
</tbody>
</table>

9.1.6 Study 2 - Liquid Diets for Intragastric Infusion: Mineral Content

Intragastric diets were prepared using the following mineral stock solutions:

<table>
<thead>
<tr>
<th>Mineral Stock Solution</th>
<th>% Solution (w/v)</th>
<th>Volume/L Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>17.33%</td>
<td>40 ml</td>
</tr>
<tr>
<td>CaCl₂.H₂O</td>
<td>43.39%</td>
<td>20 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>19.54%</td>
<td>8 ml</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>4.34%</td>
<td>4 ml</td>
</tr>
<tr>
<td>MnSO₄.H₂O</td>
<td>0.61%</td>
<td>4 ml</td>
</tr>
<tr>
<td>KI</td>
<td>0.0215%</td>
<td>4 ml</td>
</tr>
<tr>
<td>(NH₄)Mo₇O₂₄•4H₂O</td>
<td>0.0125%</td>
<td>4 ml</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>1.22%</td>
<td>4 ml</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>0.255%</td>
<td>4 ml</td>
</tr>
<tr>
<td>Fe(NH₃)₂-citrat</td>
<td>3.115%</td>
<td>4 ml</td>
</tr>
</tbody>
</table>

11 Lactalbumin hydrolysate replaces casein used in the oral diets as the source of protein.
9.1.7 Study 2 - Liquid Diets for Intragastric Infusion: Mineral Concentration

The final concentration of minerals in the intragastric liquid diets was as follows:

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Concentration (weight/L diet)</th>
</tr>
</thead>
<tbody>
<tr>
<td>calcium</td>
<td>2.4 g</td>
</tr>
<tr>
<td>phosphorous</td>
<td>1.6 g</td>
</tr>
<tr>
<td>potassium</td>
<td>2.0 g</td>
</tr>
<tr>
<td>magnesium</td>
<td>35.1 mg</td>
</tr>
<tr>
<td>sodium</td>
<td>614.0 mg</td>
</tr>
<tr>
<td>chlorine</td>
<td>5.1 g</td>
</tr>
<tr>
<td>iron&lt;sup&gt;12&lt;/sup&gt;</td>
<td>2.5 g</td>
</tr>
<tr>
<td>sulfur</td>
<td>57.3 mg</td>
</tr>
<tr>
<td>manganese</td>
<td>7.9 mg</td>
</tr>
<tr>
<td>copper</td>
<td>12.5 mg</td>
</tr>
<tr>
<td>iodine</td>
<td>650.0 μg</td>
</tr>
<tr>
<td>zinc</td>
<td>4.9 mg</td>
</tr>
<tr>
<td>molybdenum</td>
<td>266.0 μg</td>
</tr>
</tbody>
</table>

<sup>12</sup> Supplied as carbonyl iron.
9. APPENDIX 2

9.2 Effect of Propylthiouracil on Thyroid Hormones and NADPH-Cytochrome P450 Reductase in Rats: A Dose Response Study

The following study examined the dose-response relationship of PTU with respect to circulating thyroid hormones and hepatic microsomal NADPH-cytochrome P450 reductase (CYP-reductase) activity in rats. This pilot study was used to establish the optimal dose range of PTU for in vivo studies presented in this thesis.

Male Wistar rats (n=2 per group) weighing 300-350 g were administered PTU for 8 consecutive days by: (a) continuous intragastric infusion (method described in 4.2.2) as a component of the liquid diet (dose = 100 μg/kg, 200 μg/kg or 2 mg/kg/day); or (b) by oral intubation as single daily bolus dose of 20 mg/kg/day (as described in 3.2.3) in chow-fed rats. An additional group of untreated chow-fed rats was included as a control group.

Thyroid hormones were determined (see 3.2.5.1) as total T₄ and free thyroxine index, in serum samples obtained prior to treatment (Day 1) and again after 8 days of PTU administration. CYP-reductase activity in liver microsomes (see 3.2.5.5) was determined at the end of the PTU treatment period.

Serum T₄ levels (Figure 20) and free thyroxine index (Figure 21) were substantially reduced by (i) continuous intragastric administration of PTU at doses of 200 μg/kg/day or greater (67-75% decrease); and (ii) by PTU delivered by oral intubation as a single daily bolus dose of 20 mg/kg/day (approximately 80% decrease).

A dose-dependent reduction in CYP-reductase activity (5-29%) was observed in rats receiving PTU by continuous intragastric infusion at doses of 100 μg/kg - 2 mg/kg (Figure 22). Administration of PTU by oral intubation at a dose of 20 mg/kg/day resulted in an even greater reduction (40%) in CYP-reductase activity.
The results of this study provide useful preliminary information regarding the dose, interval and route of administration of PTU required to lower circulating thyroid hormone levels and hepatic microsomal CYP-reductase activity in rats.
Figure 20: Pilot study on the effect of PTU on serum T₄ concentration in rats. Serum samples were analyzed before (open bars) and after 8 consecutive days of PTU administered by (i) continuous intragastric infusion or (ii) oral intubation as a single daily bolus dose. Vertical bars indicate mean ± SEM.
Figure 21: Pilot study on the effect of PTU on serum free thyroxine index in rats. Serum samples were analyzed before (open bars) and after 8 consecutive days of PTU administered by (i) continuous intragastric infusion or (ii) oral intubation as a single daily bolus dose. Vertical bars indicate mean ± SEM.
Figure 22: Pilot study on the effect of propylthiouracil (PTU) on hepatic microsomal cytochrome P450-reductase activity in rats. Liver samples were analyzed after 8 consecutive days of PTU administered by (i) continuous intragastric infusion or (ii) oral intubation as a single daily bolus dose. Vertical bars indicate mean ± SEM.
9. APPENDIX 3

9.3 Effect of Propylthiouracil on Hepatic Non-Heme Iron Content in Chronic Ethanol-Treated Rats

Studies presented in this thesis indicate that propylthiouracil can significantly reduce hydroxyl radical formation in rat liver microsomes, an effect which is likely to be mediated by a decrease in the levels of cytochrome P450-reductase (see Study 1). An alternate explanation for the reduction in OH· formation in PTU-treated rats stems from prior findings that PTU significantly decreases hepatic iron content in rats (Al-Khayat et al., 1982) and mice (Gonzalez-Reimers et al., 1989). Since iron serves as a catalyst for the formation of OH· by the Haber-Weiss reaction, it is possible that reduced hepatic iron content may contribute to the decreased rate of OH· generation in PTU-treated rats. To test this possibility, hepatic non-heme iron levels were examined in ethanol-treated and control rats following treatment with PTU.

9.3.1 Method

9.3.1.1 Animal Treatment: Female Wistar rats (Charles River, Quebec) weighing 125-135 g were given continuous access for 29 days to a liquid diet containing ethanol or an isocaloric diet (as described in 1.3.1.1). Two additional groups of ethanol-treated and control rats also received PTU (average dose = 24.3 ± 0.6 mg/kg/day) in the liquid diet during the last 10 days of ethanol treatment. On the final day of the experiment, rats were anaesthetized with ether and the livers perfused in situ with 30 ml of ice cold phosphate buffered saline, immediately frozen in liquid nitrogen and stored at -70°C prior to preparation of tissue homogenates.

9.3.1.2 Preparation of Liver Homogenates: The following method of liver tissue preparation is based on that described by Smith et al. (1979). A portion of liver (150-400 mg wet
weight) was homogenized in 1 ml of distilled water pre-treated with Chelex-100 resin to removing contaminating iron (Buettner, 1988). The residual iron content of Chelex-treated water was below the detection limit of the assay (<1.5 μg/g). A 1 ml aliquot of homogenate was combined with 1 ml of 1.35 M trichloroacetic acid and heated at 90 °C for 15 minutes in test tubes covered with glass marbles to prevent evaporation. Samples were then centrifuged at 11000 x g for 10 minutes and the supernatants stored at -70 °C.

9.3.1.3 Non-Heme Iron Analysis: Iron content was determined by acetylene flame atomic absorption spectroscopy using a Varian Model 875 atomic absorption spectrophotometer equipped with a graphite furnace Model GTA 95 and autosampler (Lugowski et al. 1991). Iron content in samples was determined by comparison with standards containing known amounts of iron (prepared in 0.0625 M trichloroacetic acid). Bovine liver (National Institute of Standards and Technology Reference Material 1577b) containing 184 ± 15 μg iron/g liver (manufacturers specification) was used as a reference standard. All samples and standards were diluted 1:10 in 0.6% nitric acid prior to analysis.

9.3.2 Summary of Findings

Iron content determined in bovine liver reference standards (197.3 ± 0.5 μg/g) was within the limits specified by the manufacturer (185 ± 15 μg/g, intra-assay variation < 0.5%), thus establishing the accuracy and precision of the analytical method. Iron content in control rat liver samples (153.5 ± 19.0 μg/g liver) was near the range of 200-400 μg/g reported in previous studies (Smith et al., 1974). Iron content was not significantly altered in rats treated with ethanol, PTU, or ethanol + PTU (Figure 23). The discrepancy between the results obtained and those reported in previous investigations on the effect of PTU on hepatic iron levels (Al-Khayat et al.,
1982; Gonzalez-Reimers et al., 1989) are likely due to differences in the dose and method of administration, however this cannot be confirmed as the exact dose of PTU used in those studies was not reported. Since PTU did not alter the hepatic concentration of non-heme iron in rats, our earlier observation that PTU reduces microsomal OH- formation appears to be solely attributable to the lower levels of CYP-reductase, rather than decreased availability of catalytic iron in the liver.
Figure 23: Effect of propylthiouracil on hepatic non-heme iron content in ethanol-treated and control rats. PTU (average dose = 24.3 ± 0.6 g/kg/day) was administered for 10 days to rats receiving either ethanol-diet or control diet for 29 days. Hepatic non-heme iron content was determined by acetylene flame atomic absorption spectroscopy. Vertical bars indicate mean ± SEM (n=7 per group). Horizontal bars indicate significant differences between groups as determined by post-hoc Duncan's Test.
9. APPENDIX 4

ANTISENSE INHIBITION OF MYELOPEROXIDASE: A NOVEL STRATEGY FOR THE TREATMENT OF INFLAMMATORY TISSUE INJURY.

9.4 Myeloperoxidase Inhibitors: Potential Applications for the Therapy of Inflammation

The accumulation of neutrophils in infected tissues constitutes the body's first line of immunological defense against invading microorganisms. However, neutrophil-derived oxidants and proteolytic enzymes, which mediate the antimicrobial action of neutrophils, can also promote injury to surrounding healthy tissue. The identification of drugs that inhibit the neutrophil enzymes catalyzing the formation of free radicals - i.e., NADPH oxidase and myeloperoxidase - is a strategy which may be useful in the design of novel pharmacological therapies for inflammatory tissue injury.

NADPH oxidase catalyzes the formation of superoxide anion (1), which dismutates to H$_2$O$_2$ (2) and generates the highly toxic hydroxyl radical (OH') via the iron-catalyzed Haber-Weiss reaction (3).

\[
\begin{align*}
2O_2 + \text{NADPH} &\rightarrow 2O_2^- + \text{NADP}^+ + H^+ \\
2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2 \\
O_2^- + H_2O_2 &\rightarrow [Fe^{2+}] \rightarrow O_2 + OH^- + OH^-
\end{align*}
\]

Inhibitors of NADPH oxidase are of therapeutic interest, since they may be capable of preventing tissue injury that may result from OH' generated by neutrophils. However, NADPH oxidase plays an integral role in preventing host tissue infection and genetic deficiency of NADPH oxidase (chronic granulomatous disease) is associated with high rates of infection by Staphylococcus aureus, Salmonella, Aspergillus and Candida (Tauber et al., 1983) and shortened life expectancy (Curnutte, 1993). NADPH oxidase inhibitors are therefore not ideal anti-inflammatory drugs, since they have the potential to cause significant impairment of host immunity.
Myeloperoxidase (MPO) is an enzyme present in neutrophil azurophilic granules that catalyzes the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions:

\[ \text{H}_2\text{O}_2 + \text{Cl}^- + \text{H}^+ \rightarrow \text{HOCl} + \text{H}_2\text{O} \quad (4) \]

Hypochlorous acid can promote tissue injury by (i) direct toxicity towards cells, (ii) the generation of hydroxyl radicals or (iii) causing the inactivation of \( \alpha\)-1-proteinase inhibitor (A1PI), an endogenous protein that normally protects tissues from injury by neutrophil-derived proteolytic enzymes. The use of pharmacological agents to inhibit MPO may therefore be another potentially effective strategy in the therapy of inflammatory tissue injury. The observation that genetic deficiency of MPO in humans is not associated with a high incidence of life-threatening infections (Parry et al., 1981), as occurs with NADPH oxidase deficiency, suggests that MPO inhibitors may be used to treat inflammation without greatly compromising host immunity.

The ability to scavenge HOCl generated by MPO is a mechanism which has been proposed to contribute to the anti-inflammatory properties of a variety of therapeutic agents, including non-steroidal anti-inflammatory drugs (Shacter et al., 1991; Dallegri et al., 1992a; 1992b; 1990a; 1990b), aminoglycoside antibiotics (Cantin and Woods, 1993) and anti-ulcer medications (Van Zyl et al., 1993). Drugs that prevent the formation of HOCl by directly inhibiting MPO may also be effective in the therapy of inflammation. The studies presented in this thesis indicate that inhibition of MPO is likely to be an important mechanism contributing to the cytoprotective effects of PTU against liver injury induced by ethanol and other hepatotoxins (Israel et al., 1975; Raheja et al., 1982; Orrego et al., 1976; 1987; Oren et al., 1996; Cooper et al., 1984) and the efficacy of PTU therapy for psoriasis (Elias et al., 1994). In the treatment of alcoholic liver disease, PTU therapy is not associated with an increased incidence of adverse side effects (Orrego et al., 1994). However, in the treatment of Grave’s hyperthyroidism, where higher doses (300-1000 mg vs. 300 mg) may be used (Cooper, 1984), PTU may produce side effects such as
hypothyroidism and rarer adverse reactions including agranulocytosis and systemic lupus erythematosus (Uetrecht, 1990). The potential to produce these unwanted effects may ultimately limit the use of current antithyroid drugs in the therapy of inflammation. One means of overcoming this limitation would be to develop chemically-modified analogs of PTU which have reduced systemic toxicity, while retaining the anti-inflammatory properties of the parent molecule. Several observations suggest however that achieving such specificity may not be feasible. PTU is metabolized by neutrophil MPO, resulting in the formation of electrophilic intermediates which in turn inactivate the enzyme (Lee et al., 1990). A similar mechanism appears to mediate the antithyroid effects of PTU. Reactive PTU metabolites are generated by, and are likely inhibitors of, thyroid peroxidase - an enzyme involved in the synthesis of thyroid hormones which has a polypeptide sequence very similar to that of MPO (Kimura et al., 1988). PTU-induced agranulocytosis and lupus are also thought to be caused by metabolism of PTU in neutrophils to electrophillic intermediates which are toxic to neutrophils or their bone marrow precursors (Waldhauser and Uetrecht, 1991; Uetrecht et al., 1990). Since the formation of electrophillic metabolites may be a critical mechanism underlying the cytoprotective effect of PTU (i.e., inhibition of MPO) as well as the drugs undesired side effects, chemical modifications of the PTU parent molecule intended to limit reactive metabolite formation, would likely also result in a loss of MPO inhibitory activity and cytoprotective efficacy.

9.5 Targeting Gene Expression: Antigene (Triple-Helix) and Antisense Oligonucleotides

The mechanism of action of many traditional pharmacotherapeutic agents involves inhibiting the function of a protein or enzyme involved in disease pathogenesis, by binding to and inactivating its active site. As discussed above in reference to PTU, such mechanisms of action often lack specificity and produce undesirable effects, limiting the efficacy of therapy. An
alternative to using protein-targeted drugs to modulate enzyme activity is to inhibit the production of the protein altogether by using gene-targeted drugs (i.e., oligonucleotides) which bind to specific sites on the nucleic acids (DNA and RNA) that direct protein synthesis. In order for a protein to be synthesized, the gene that codes for it must be transcribed or copied from double-stranded DNA into single-stranded mRNA, which is then transported to the cytoplasm and translated by ribosomes into the specified protein. During the translation step, a series of three mRNA nucleotides, known as a codon, directs the addition of a specific amino acid to the growing protein chain. In theory, arrest of transcription or translation at any point would prevent the protein product of a gene from being expressed. If the nucleotide sequence of a gene is known, an oligonucleotide can be synthesized which recognizes and binds to the complementary sequence within the targeted DNA or RNA, thereby arresting protein synthesis. Because the basis of this idea stems from the specificity of Watson-Crick base pairing (i.e., cytosine-guanine and adenine-thymine), such therapies are in theory, exquisitely specific and may therefore reduce the problem of systemic toxicity.

Two basic approaches have been used in the development of nucleic acid binding drugs to inhibit protein synthesis. Use of an oligonucleotide to stall transcription is known as the triplex or triple-helix (antigene) strategy, since the oligonucleotide winds around double-helical DNA, forming a three-stranded helix. The second strategy, referred to as antisense, involves the use of oligonucleotides (antisense strands) that bind complementary sequences in mRNA (sense strand) to arrest translation of the coded protein. At present, antigene sequences can bind only to a limited number of DNA duplex strands and may lack the sequence-specific potential of antisense oligonucleotides. This limited potential is due to the fact that the antigene sequences do not bind to the targeted DNA via base pairing. Rather, the antigene sequences bind to the duplex DNA to form a triple helix through what are called Hoogsteen base pairs. Hoogsteen base pairing occurs
predominantly at duplex DNA sites that are rich in A-T bonds or in G-C bonds (Helene, 1991; Cohen, 1991). Since this type of sequence does not lend itself to sequence-specific binding, the specificity of the antigenic sequence can be compromised, resulting in decreased therapeutic potential (Putnam, 1996). Furthermore, it has been estimated that the frequency with which such sequences occur in the mammalian genome is too low to consistently provide reasonable targets in gene promoters (Behe, 1987; Maher, 1996). Despite these potential limitations, several investigators have successfully inhibited enzyme gene expression using triplex forming oligonucleotides. In contrast with the triple-helix technique, a higher degree of sequence-specific inhibition of protein synthesis may be achieved using antisense oligonucleotides and the application of such therapies in human diseases holds the promise of potentially dramatic therapeutic advances. The remainder of the present work focuses on the potential applications of antisense oligonucleotides as inhibitors of MPO.

9.6 *General Considerations in the Design of Antisense Oligonucleotides*

Several factors have been identified which ultimately determine the effectiveness of antisense oligonucleotides in arresting protein synthesis (Putnam, 1996; Bennett and Schwartz, 1995; Stein and Chiang, 1993). First, the antisense agent should be stable *in vivo*, both intracellularly and extracellularly. Second, the antisense agent must be capable of entering cells and binding to the target sequence with relatively high affinity, at concentrations that do not exert significant toxicity to the cell. Third, hybridization to the target sequence should induce suppression of gene expression of the target, and to no other nucleic acid sequences, or to intracellular proteins or lipids. Based on these criteria, a number of physicochemical characteristics of the oligonucleotide are considered when a sequence is selected for use as an antisense agent. In particular, the optimal length, target gene sequence, stability and uptake of the oligonucleotide, and nonspecific effects due to the agent all must be addressed.
9.6.1 Antisense Oligonucleotide Chain Length

The hybridization of base sequences between nucleic acids is very specific; only the complementary base (C-G, A-T) should be bound. Because the mRNAs of related proteins often have areas lacking significant homology, this specificity of base pairing means that an antisense sequence of bases should target only a single mRNA, without affecting the mRNAs of other genes. As evidence of this specificity, studies have shown that mRNAs can discriminate between oligonucleotides that differ by one or two bases (Wang et al., 1985; Holt et al., 1988; Bennett et al., 1994).

An oligonucleotide must be long enough to be unique to the target mRNA but not so long that it binds to multiple mRNA species nonspecifically. Based on the complexity of the human genome, with approximately 3 to 4 million bases, it is statistically unlikely that a specific sequence of 17 or more nucleotides will occur more than once (Uhlmann and Peyman, 1990) and the shortest sequence required for recognition of a unique sequence is 12 to 15 bases (Lewin, 1990). Oligos with shorter chain lengths are more likely to bind with complementary sequences in non-targeted mRNA species and thereby produce non-specific effects. In practice, most studies have used oligonucleotides of 15 to 30 bases. The longer the sequence, the greater the affinity of the antisense strand for its targeted sequence. However, extremely long antisense oligonucleotides are more likely to also bind to non-targeted nucleotide sequences and lead to nonspecific effects. Furthermore, longer chain oligos also show decreased cellular uptake and are more expensive to synthesize than shorter oligos.
9.6.2 mRNA Target Sequence

Although the precise mechanisms by which antisense oligonucleotides reduce target mRNA and protein levels within the cell are imperfectly understood, two main mechanisms have been postulated. First when targeted to the 5' region (particularly near the cap and AUG initiation codon), oligomers act as a physical block to binding of the initiation complex, scanning of the 5' leader by the 40 S subunit or the assembly of a functional 80 S complex at the AUG initiation codon. Second antisense oligonucleotides induce the cleavage of mRNA by the nuclease RNase H, which is widely present in mammalian cells and specifically recognizes DNA-RNA duplexes (Wagner and Nishikura, 1988). The presence of RNAase H leads to irreversible effects of antisense oligos since the cleaved mRNA can no longer support translation. Antisense inhibition of translation mediated by RNAase H has been proposed to account for the finding that some antisense oligomers complementary to the coding region and 3' untranslated region of the target mRNA may be equally or more effective than oligomers targeting the 5' region.

Because virtually any region of an mRNA may be a suitable antisense target the design of oligomer sequences is an informed guess at best, and many sequences are usually tested before sequences are chosen that exert maximal suppression of target gene expression. (Chiang et al., 1991). A review of past studies reporting successful antisense inhibition of protein synthesis, indicates that a specific nucleotide motif - CCCT (reading 3' to 5') - is often contained in the antisense structure (Tu, personal communication). Antisense agents containing this motif would be expected to bind to gene targets containing the complementary sequence GGGA (reading 5' - 3'). These findings provide a useful strategy for identifying both suitable mRNA target sequences (i.e., those containing the 5'-3' GGGA motif) and for the design of complementary antisense oligomers (i.e., those containing the 3'-5' CCCT motif).

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9.6.3 Antisense Concentration in Target Cells

A critical parameter determining antisense efficacy is the concentration of the antisense oligomer achieved within cells, which in turn is dependent upon a) the penetration of the oligomer across cell membranes and b) the degradation of the oligomer molecule by endogenous DNAases.

The exact mechanism of oligonucleotide entry into cells is unclear. Studies using fluorescent acridine-labeled oligonucleotides have suggested that uptake of oligonucleotides is by a mechanism consistent with receptor-mediated endocytosis, and two surface proteins (34 and 80 kD) have been identified that may mediate the process (Loke et al., 1989; Yakubov et al., 1989). Uptake of antisense oligomers is generally an inefficient process, (Spiller and Tidd, 1992) but it can be enhanced by complexing the oligonucleotide sequence with liposomes (Juliano and Akhtar, 1992; Clarenc et al., 1993; Loke et al., 1988; Bennett et al., 1992; Capaccioli et al., 1993). Use of liposomes masks the negative charge present on many types of oligonucleotides, and may thus allow diffusion across the cell membrane. Liposome encapsulation of oligos also results in a more diffuse pattern of oligo distribution within cells, as compared to unencapsulated oligos, which reside mainly in localized intracytoplasmic vesicles (Thiery and Dritschillo, 1992).

The instability of oligonucleotides has been a significant problem in their use in vitro and their potential use in vivo. Among the first antisense compounds to be studied consisted of unmodified oligodeoxynucleotides. These agents were ineffective due to their rapid digestion by endogenous intracellular exonucleases which attack the phosphodiester linkage between nucleotides (Wickstrom, et al., 1988). Several structural modifications of the oligo backbone have been developed to reduce susceptibility to nuclease degradation. Replacing one of the nonbridging oxygen atoms in the internucleotide bond with a methyl group to form methyl phosphonate oligos (MP) eliminates the net negative charge of the molecule and aids in preventing nuclease digestion. While such a modification enhances resistance to degradation, it greatly decreases solubility as
compared to unmodified oligodeoxyribonucleotides. Phosphorothioate oligos (PS), in which one of the non-bridging oxygen atoms is replaced with sulfur, are significantly less susceptible to nuclease digestion than unmodified oligos but maintain better solubility characteristics than MP oligos. At the present time, modified oligonucleotides in general and phosphorothioates in particular are widely considered to be the most promising agents for therapeutic use (Stein and Cohen, 1989).

9.6.4 Pharmacokinetics and Tissue Distribution of Phosphorothioate Oligos

The in vivo pharmacokinetics of PS oligos administered by various routes have been characterized in several mammalian species. The concentration of PS oligos in the circulation following intravenous administration in mice, rats, monkeys and humans exhibits a biphasic profile. In humans, the mean distribution-phase half-life of i.v. administered PS oligos is 0.18 hours, and the mean elimination-phase half-life is 26.71 hours. Elimination occurs primarily through metabolism and excretion with roughly half the dose being eliminated (as degraded oligonucleotide) in the urine after 24 hours and 70% (as degraded oligonucleotide) after 96 hours. In rats the tissue distribution and metabolism of PS oligos is similar whether given by i.v., i.p. or s.c. routes of administration, with highest levels detectable in liver, kidneys and bone marrow (Cossum et al., 1993).

9.6.5 Non-specific Effects of Antisense Drugs

PS oligos have been reported to produce a number of side effects in monkeys and rats, such as complement activation and interference with blood clotting factors (Yu et al., 1996, Shaw et al., 1997). It is presently not known whether similar non-specific effects occur in humans treated with PS oligos. The mechanism mediating these non-specific effects is not precisely known
but may be related to either the presence of sulfur in the oligo backbone or to the chain length of the oligo. The longer the oligonucleotide the greater the chance of hybridization to nontargeted mRNAs by short sections of consecutive nucleotides. This has been used as an argument for restricting antisense oligonucleotides to 15 to 20 bases in length (Bennett and Schwartz, 1995).

Since the toxicity of PS oligos is dose-dependent, an important consideration in antisense drug delivery is to achieve sufficient concentration of oligos in target cells, while minimizing the concentration in non-targeted tissues.

9.7 Developing Antisense Inhibitors of Myeloperoxidase

9.7.1 Target Sequence

The preceding discussion highlights several factors which must be considered in order to successfully develop antisense inhibitors of MPO. An initial focus of such research would be to analyze the nucleotide sequence of the MPO gene, which has been sequenced previously (Morishita et al., 1987), and to select suitable target sequences for the design of complementary antisense oligos. Since very little is currently known regarding the ideal target sequences for antisense inhibition, designing antisense oligos is to some extent a trial and error process. A more methodical approach is possible by considering some of the guidelines for antisense drug design outlined above. For instance, antisense agents may be particularly effective when they (a) are complementary to sequences in the 5' region of target gene, and (b) contain the sequence 3'-CCCT-5', which is complementary to sequence 5'-GGGA-3' in the target gene. Oligos complementary to those regions around and including the GGGA motifs in the MPO gene offer potential as antisense inhibitors of MPO. We have determined that the MPO gene has 20 regions containing the 5'-GGGA-3' motif. Initial experiments would focus on testing the effects of a series of complementary 32-mer oligonucleotides on MPO protein and mRNA levels. These
Table 9: Myeloperoxidase Antisense Target Sequences and Complementary Antisense Sequences.

<table>
<thead>
<tr>
<th>MPO Gene Target Sequences</th>
<th>Complementary 32-mer Antisense Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>66</td>
</tr>
<tr>
<td>5'-CAGCAGCTGGCAAGGGGATAAGAGAGCAGTGA-3'</td>
<td>(1) 3'-GTCTTGGACCAGTGCTGCTCATC-5'</td>
</tr>
<tr>
<td>103</td>
<td>134</td>
</tr>
<tr>
<td>5'-CTGAGGTACAAAGGGGATGGGACGCCTCGT-3'</td>
<td>(2) 3'-GACTCCATGTTCCCTACTGCTGCTGGTCC-5'</td>
</tr>
<tr>
<td>313</td>
<td>346</td>
</tr>
<tr>
<td>5'-TCCAGCTGCTCTGGGAGGTGAGCCACCTCCTG-3'</td>
<td>(3) 3'-AGGTCGAGCAGGCCCTCCACCTGTTGGAGCA-5'</td>
</tr>
<tr>
<td>391</td>
<td>412</td>
</tr>
<tr>
<td>5'-CTACAGAGGAGCGGGGAAAGCATAAAGCAGC-3'</td>
<td>(4) 3'-GATGTCGCGGCTGGGCTTCTAGTTGGTGC-5'</td>
</tr>
<tr>
<td>681</td>
<td>712</td>
</tr>
<tr>
<td>5'-ACCGCAACCTACAGGGGATGTGCAACAACAGA-3'</td>
<td>(5) 3'-TGCCGTTGATGCGGCTACATCAGTGTGGTCT-5'</td>
</tr>
<tr>
<td>1110</td>
<td>1141</td>
</tr>
<tr>
<td>5'-GCCGGCTTGGGCGGGGAAAGCATAACACAGG-3'</td>
<td>(6) 3'-CGGCCGGAACCTTTTCTCTGCTGTAGTGCAG-5'</td>
</tr>
<tr>
<td>1354</td>
<td>1385</td>
</tr>
<tr>
<td>5'-CTGCTTCTGGCAGGGGACACCCGTTCCAGTG-3'</td>
<td>(7) 3'-GACGGAGGAGCGGCTGCCCTGTTGGGCAAGTC-5'</td>
</tr>
<tr>
<td>1411</td>
<td>1442</td>
</tr>
<tr>
<td>5'-CTGCTTCTGGCAGGGGACACCCGTTCCAGTG-3'</td>
<td>(8) 3'-GACGGAGGAGCGGCTGCCCTGTTGGGCAAGTC-5'</td>
</tr>
<tr>
<td>1459</td>
<td>1490</td>
</tr>
<tr>
<td>5'-CCTGAAACCACTAGGAGATGGGAGAGGCTCCT-3'</td>
<td>(9) 3'-GGACTTGGGATCCACCACTTACCTCTCCTCCAGA-5'</td>
</tr>
<tr>
<td>1465</td>
<td>1496</td>
</tr>
<tr>
<td>5'-CCCTAGGGATGGGAGAGGCTCCTCCAGA-3'</td>
<td>(10) 3'-GGGATCCAACCACCCCTCTCGAGATTTGCTT-5'</td>
</tr>
<tr>
<td>1528</td>
<td>1599</td>
</tr>
<tr>
<td>5'-GATCATCATTCTCAGGGACTACCTGGCCCTCG-3'</td>
<td>(11) 3'-CTAGTGGATGAGCCTGCTGTGGGAGAC-5'</td>
</tr>
<tr>
<td>1855</td>
<td>1886</td>
</tr>
<tr>
<td>5'-AGTGGATGAGATCCAGGGGAGAGGATTGCTCCAG-3'</td>
<td>(12) 3'-TCACCTACTCATTGAGGCTCAGTAAACACTCG-5'</td>
</tr>
<tr>
<td>1924</td>
<td>1955</td>
</tr>
<tr>
<td>5'-CATGACGCGGACACCCACCGGCTCCCCAG-3'</td>
<td>(13) 3'-GTACGGTCCGAGTCTGGCCGGGAGGTC-5'</td>
</tr>
<tr>
<td>2167</td>
<td>2198</td>
</tr>
<tr>
<td>5'-GTTCAGGAAAGCTCCGGGATGTTGCTCGGTTT-3'</td>
<td>(14) 3'-CAAGTCTTCCAGGGCCCTCAACTAGCCAAA-5'</td>
</tr>
<tr>
<td>2288</td>
<td>2219</td>
</tr>
<tr>
<td>5'-TGATCGGTTTGGGAGAGAAGAGGATTGGTTCT-3'</td>
<td>(15) 3'-ACTAGCCCAAACCACCCCTCCACCTCCTGGTCCACA-5'</td>
</tr>
<tr>
<td>2323</td>
<td>2354</td>
</tr>
<tr>
<td>5'-CAATCTATATCCCCGCCGACTTTGTGACAGC-3'</td>
<td>(16) 3'-GTGGAGTATAGGGGCCTGGAACAGTTGACG-5'</td>
</tr>
<tr>
<td>2374</td>
<td>2405</td>
</tr>
<tr>
<td>5'-CCTGGTTCCTGAGGGGAGAGAGCTTGGAGGC-3'</td>
<td>(17) 3'-GGACGGAAGGACCTCCCTCCGAGGAGTCCCGG-5'</td>
</tr>
<tr>
<td>2687</td>
<td>2618</td>
</tr>
<tr>
<td>5'-TTGCTCTAAGCTGGGAGAGGCTTGGAGGC-3'</td>
<td>(18) 3'-CAACAGACCTTGATCCACATCCAAAGTACACCAA-5'</td>
</tr>
<tr>
<td>2965</td>
<td>3024</td>
</tr>
<tr>
<td>5'-AGTGGTGCTTCCGTGGAGAAGTACACAGCTGAG-3'</td>
<td>(19) 3'-TCACCAAGGGACCTCCCTATTGGTGTCAGGTC-5'</td>
</tr>
<tr>
<td>3190</td>
<td>3193</td>
</tr>
<tr>
<td>5'-AAGGCAACCGTTGAGGGCACCTCCACTTCTG-3'</td>
<td>(20) 3'-TTCCGCTGGCACTACCCCTGGAGGAGACAGA-5'</td>
</tr>
</tbody>
</table>
structures and their complementary sequences in the *MPO* gene are shown in Table 9. Should any of these sequences be effective, subsequent experiments would determine the minimal chain length required to elicit the antisense effect, by testing their 20-mer oligo derivatives. Some of the potential side effects of these oligos, for example that which may occur due to cross-reactivity with the closely related *TPO* gene (Kimura et al., 1987), could be predicted by comparing the extent of homology between the antisense target sequences and other known sequences in the human genome, using the search capabilities of GenBank and BLAST\(^\text{14}\).

Initial experiments would use *in vitro* systems to evaluate the efficacy of antisense MPO inhibitors. Isolated neutrophils and many myeloid leukemia cell lines, such as HL-60 myeloblasts (Schlaifer et al., 1994), express MPO and are ideal for use in the proposed *in vitro* studies. HL-60 cells were used previously in the only study published to date on antisense oligonucleotide inhibition of MPO activity (Meyer et al., 1992). Incubation of HL-60 cells with an antisense oligonucleotide complementary to exon 1 of the *MPO* gene produced only a 50% decrease in MPO protein levels. Furthermore the effect was observed at an oligonucleotide concentration of 70 \(\mu\text{M}\), which is too high to be practical for clinical applications. The development of antisense oligos capable of inhibiting MPO activity at therapeutically relevant oligonucleotide concentrations (i.e., 1 \(\mu\text{M}\) or less), is a primary objective of the research proposed herein.

9.7.2 *Cellular Targets and Methods of Delivery*

Two potential cellular targets of MPO inhibitors include: (a) bone marrow - the site of production of neutrophils; and (b) the liver, which becomes infiltrated with neutrophils during the progression of many forms of liver disease (e.g. that induced by ethanol and other toxins).

\(^{14}\) GenBank is a genetic sequence database containing an annotated collection of all publicly available DNA sequences. BLAST or Basic Local Alignment Search Tool is an algorithm developed to search the GenBank database. These services are provided by the National Center for Biotechnology Information, National Institute of Health at their internet website: http://www.ncbi.nlm.nih.gov/Web/Search/index.html.
Because PS oligos naturally tend to accumulate in both bone marrow and the liver, these two sites represent ideal targets for antisense MPO inhibitors. Furthermore this tendency may be expected to minimize systemic toxicity associated with the effect of antisense oligos on non-targeted tissues, since relatively low doses of oligos could be used while still achieving sufficient concentrations in the target tissues. Studies should also be conducted to determine whether antisense efficacy could be enhanced, and systemic toxicity minimized, by using cationic liposomes as a delivery system for antisense MPO inhibitors. Since liposomes may themselves be toxic to cells, it will also be necessary to perform studies to determine (a) the optimal ratio of liposomes to antisense molecules and (b) the direct systemic toxicity of liposomes delivered without antisense oligos in various cell lines.

9.8 **Summary of Research Objectives**

1. The proposed studies would use (a) HL-60 cells and (b) human neutrophils as *in vitro* test systems to evaluate the efficacy of experimental PS antisense molecules on MPO protein and mRNA expression. These studies would initially examine a series of 32-mer antisense oligonucleotides complementary to: (a) the regions in the *MPO* gene containing GGGC motifs; and (b) various areas in the 5' region of the *MPO* gene. Upon identification of effective 32-mer antisense oligonucleotides, the second phase of the study will examine the effect of the corresponding 20-mer oligonucleotide derivatives to obtain the minimal sequence identities. In order to assess non-sequence specific effects, appropriate control sequences for each antisense oligonucleotide will also be examined. These would include oligonucleotide analogs of the same chain length and nucleotides, but with the nucleotide sequence reversed or randomly scrambled. These studies will simultaneously yield important information on the mechanism of action of antisense inhibitors of MPO. Specifically, oligonucleotides which decrease MPO mRNA levels are
likely to be acting through a mechanism that involves enhanced mRNA degradation by endogenous RNAse’s, while oligonucleotides that lower MPO protein levels but do not affect MPO mRNA levels can be assumed to be acting solely through translational arrest (i.e., by binding to the 5’ region and physically blocking mRNA translation).

2. Studies have been proposed to evaluate whether MPO antisense efficacy can be improved by administration of oligonucleotides with cationic liposomes. In order to accomplish this, antisense oligonucleotides would be delivered with varying concentrations of cationic liposomes, and the effect on MPO protein and mRNA levels determined. The toxicity of liposomes to target cells would also be simultaneously assessed using conventional methods, such as trypan blue exclusion.

3. In order to further clarify the pharmacokinetic properties of antisense oligonucleotides, studies would be conducted in vitro (i.e., HL-60 cells and human neutrophils) and in vivo (rats) to determine the rate of uptake and elimination of radiolabeled oligonucleotides. For in vivo studies, various tissues and fluids will be examined including circulating neutrophils, plasma, liver, kidney, brain, skin bone marrow and urine. Various routes of antisense oligonucleotide administration would also be used (i.e., i.v., oral and topical). These studies would also compare the pharmacokinetics profiles of antisense oligonucleotides delivered with and without cationic liposomes.

4. Non-specific effects of antisense oligonucleotides can be predicted initially by comparing antisense sequences with those of other known genes in the GenBank database. Subsequent in vivo studies will be performed to determine the effect of antisense oligonucleotides
on the protein levels of thyroid peroxidase and eosinophil peroxidase, which are closely related to MPO. In addition, systemic toxicity of MPO antisense agents would be assessed in rodents and primates.

5. The final phase of discovery would involve demonstrating the ability of MPO antisense oligonucleotides to minimize tissue damage. This would be assessed in rat models of liver injury (i.e., induced by chronic ethanol, galactosamine, endotoxin, carbon tetrachloride) and skin inflammation.

6. The ultimate aim of the studies previously outlined is to identify antisense MPO inhibitors which could be considered for clinical trials in the therapy of various human inflammatory diseases. Alcoholic liver disease and psoriasis are conditions which are most likely to be treatable using these drugs. In addition, antisense MPO inhibitors may also be effective in a variety of other conditions associated with neutrophil-mediated tissue injury, such as rheumatoid arthritis, emphysema, periodontitis, ischemia-reperfusion and organ transplantation injury.