"ETHANOL-INDUCED FATTY LIVER. PROTECTIVE ACTION OF (+)-CATECHIN COMPOUNDS"

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

in The University of Surrey

by

PETER ROBERT RYLE

Greenwich District Hospital
Vanbrugh Hill
London SE10 9HE

August 1986
To my Parents
SUMMARY

The aim of the work presented in this thesis was to assess the protective properties of the bioflavanoid drug, (+)-catechin, and its palmityl ester, 3-palmitoyl-(+)-catechin, against ethanol hepatotoxicity (ie: fatty liver) in the rat. In initial experiments, both (+)-catechin compounds were found to protect against the hepatic lipid accumulation (mainly triglyceride) after acute ethanol dosing, and after long-term feeding of ethanol in a liquid diet. In the latter situation, 3-palmitoyl-(+)-catechin was significantly more effective than (+)-catechin itself at preventing fatty liver, probably as a result of its greater lipid solubility and longer half-life in the liver tissue.

Published work suggested two possible mechanisms of action for the (+)-catechin compounds against ethanol hepatotoxicity. Firstly, the ability of the drugs to correct the elevated hepatic NADH:NAD ratio (redox-state) after ethanol dosing may limit steatosis. Secondly, free radical scavenging properties may prevent liver injury occurring as a result of ethanol-induced lipid peroxidation. Acute experiments were performed which confirmed that the (+)-catechin compounds corrected the redox-state change after acute ethanol administration, but subsequent studies in which correction of the redox-state by Naloxone or Methylene Blue was found to have little influence on ethanol-induced steatosis, suggested that this was not the mechanism of action of the drugs. Synthetic antioxidants (free radical scavengers) were found to prevent both acute and chronic ethanol-induced fatty liver, under the same experimental conditions as...
those under which the (+)-catechin compounds afforded protection, without reversing the redox-state change after ethanol dosing. 3-Palmitoyl-(+)-catechin was then shown to prevent ethanol-induced hepatic lipid peroxidation (measured as mitochondrial diene conjugates and liver malonaldehyde levels) after acute ethanol dosing, at the same time as preventing triglyceride accumulation. As other effects of the compounds which might influence fat accumulation after ethanol were excluded (eg: inhibition of ethanol metabolism or lowering of liver acetaldehyde concentrations), it was concluded that the (+)-catechin compounds protect against alcoholic fatty liver in rats by inhibiting ethanol-induced lipid peroxidation, and the possible consequence of the latter (ie: mitochondrial damage and impaired fatty acid oxidation), rather than acting through modulation of the redox-state. The findings here cast doubts on the commonly-quoted 'redox-state' mechanism for fatty liver production by ethanol, and support the lipid peroxidation hypothesis for alcoholic liver injury.
CONTENTS

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td>vii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>viii</td>
</tr>
<tr>
<td>Publications</td>
<td>ix</td>
</tr>
<tr>
<td>Chapter 1. INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1. THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE</td>
<td></td>
</tr>
<tr>
<td>1.1.1. Animal Models of Alcoholic Liver Disease</td>
<td>3</td>
</tr>
<tr>
<td>1.2. BIOCHEMICAL MECHANISMS OF ALCOHOLIC LIVER INJURY</td>
<td></td>
</tr>
<tr>
<td>1.2.1. Relationship Between Acute and Chronic Fatty Liver</td>
<td>6</td>
</tr>
<tr>
<td>1.2.2. The Metabolism of Ethanol: Its Role in Fatty Liver Production</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3. Possible Metabolic Consequences of the Hepatic Redox-State Change After Ethanol: Relationship to Fatty Liver Production</td>
<td>12</td>
</tr>
<tr>
<td>1.2.4. Assessment of Possible Role of Redox-State Related Effects in Hepatic Steatosis</td>
<td>17</td>
</tr>
<tr>
<td>1.2.5. Role of Acetaldehyde in the Pathogenesis of Alcoholic Fatty Liver</td>
<td>22</td>
</tr>
<tr>
<td>1.2.6. Ethanol-Induced Hepatic Lipid Peroxidation</td>
<td>31</td>
</tr>
<tr>
<td>1.3. OTHER POSSIBLE MECHANISMS OF FATTY LIVER PRODUCTION BY ETHANOL</td>
<td></td>
</tr>
<tr>
<td>1.3.1. Role of Ethanol-Derived Acetate</td>
<td>43</td>
</tr>
<tr>
<td>1.3.2. Increased Peripheral Lipolysis</td>
<td>44</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.3.3. Induction of Specific Enzyme Activities</td>
<td>44</td>
</tr>
<tr>
<td>1.3.4. Increased Hepatic Blood Flow</td>
<td>46</td>
</tr>
<tr>
<td>1.3.5. Hypoxia and Disturbances in Thyroid Hormone Metabolism</td>
<td>47</td>
</tr>
<tr>
<td>1.3.6. Disturbances in Essential Fatty Acid and Prostaglandin Metabolism</td>
<td>51</td>
</tr>
<tr>
<td>1.3.7. Carbohydrate Deprivation and Alcoholic Fatty Liver</td>
<td>54</td>
</tr>
<tr>
<td>1.4. MECHANISMS OF ALCOHOL-INDUCED FATTY LIVER: CONCLUDING REMARKS</td>
<td>56</td>
</tr>
<tr>
<td>1.5. STUDIES ON HEPATOPROTECTIVE AGENTS IN ALCOHOLIC FATTY LIVER</td>
<td>58</td>
</tr>
<tr>
<td>1.5.1. Substances Other Than (+)-Catechin Compounds</td>
<td>59</td>
</tr>
<tr>
<td>1.5.2. (+)-Catechin (Cyanidanol-3) and 3-Palmitoyl-(+)-Catechin</td>
<td>67</td>
</tr>
<tr>
<td>1.6. PROPOSED STUDIES</td>
<td>79</td>
</tr>
<tr>
<td>Chapter 2 MATERIALS AND METHODS</td>
<td>82</td>
</tr>
<tr>
<td>2.1. CHEMICALS, DIETS AND REAGENTS</td>
<td>82</td>
</tr>
<tr>
<td>2.2. INDUCTION OF ACUTE AND CHRONIC ETHANOL-INDUCED FATTY LIVER IN THE RAT</td>
<td>85</td>
</tr>
<tr>
<td>2.2.1. Acute Studies</td>
<td>85</td>
</tr>
<tr>
<td>2.2.2. Chronic Ethanol Intake Studies</td>
<td>88</td>
</tr>
<tr>
<td>2.3. ASSAYS FOR LIVER LIPID CONTENT</td>
<td>95</td>
</tr>
<tr>
<td>2.3.1. Total Lipid Assay</td>
<td>95</td>
</tr>
<tr>
<td>2.3.2. Liver Triglyceride Assay</td>
<td>96</td>
</tr>
<tr>
<td>2.4. LIVER METABOLITE ASSAYS</td>
<td>99</td>
</tr>
<tr>
<td>2.4.1. Sample Collection</td>
<td>99</td>
</tr>
</tbody>
</table>
2.4.2. Sample Extraction 99
2.4.3. ATP Assay 100
2.4.4. Pyruvate Assay 101
2.4.5. Acetoacetate Assay 101
2.4.6. Lactate Assay 102
2.4.7. 3-Hydroxybutyrate Assay 103
2.4.8. α-Glycerophosphate Assay 103
2.4.9. Calculation of Results from Metabolite Assays 104

2.5. METHODS FOR INVESTIGATING HEPATIC LIPID UTILISATION AFTER ACUTE ETHANOL ADMINISTRATION 106

2.5.1. Hepatic Uptake and Incorporation of [U-14C]-Palmitic Acid 106
2.5.2. Oxidation of [1-14C] Palmitic Acid by Rat Liver Slices 109
2.5.3. Krebs-Hanseleit Bicarbonate Buffer (pH = 7.4; calcium free) 111
2.5.4. Label Solution ([1-14C] Palmitic Acid; 2.5 µCi/ml; 5 mM) 111
2.5.5. Other Determinations 112
2.5.6. Validation of Fatty Acid Oxidation Experiments 114
2.5.7. Scintillation Counting 117

2.6. GAS CHROMATOGRAPHIC ANALYSIS OF ACETALDEHYDE AND ETHANOL IN RAT BLOOD, SERUM AND LIVER SAMPLES 117

2.6.1. Sample Collection and Preparation 118
2.6.2. Gas Chromatography 119
2.6.3. Validation of Methodology

2.7. ENZYMATIC DETERMINATION OF ETHANOL IN BLOOD AND SERUM SAMPLES

2.8. ASSAYS FOR IN VITRO ANTIOXIDANT ACTIVITY OF DRUGS

2.8.1. Assay for Ability to Scavenge Superoxide Anions

2.8.2. Assay for Ability to Scavenge Hydroxyl Free Radicals

2.9. ASSAYS FOR HEPATIC LIPID PEROXIDATION

2.9.1. Measurement of Hepatic Lipid Peroxidation by the Presence of Diene Conjugation in Microsomal and Mitochondrial Lipids

2.9.2. Measurement of Liver Malonaldehyde Levels by the Thiobarbituric Acid Reaction

2.9.3. Measurement of Liver Reduced Glutathione (GSH) Concentrations

2.10. MISCELLANEOUS ASSAYS

2.10.1. Serum Glutamate Dehydrogenase Activity (GLDH)

2.10.1. Statistical Analysis

Chapter 3  PROTECTIVE EFFECTS OF (+)-CATECHIN COMPOUNDS AGAINST ACUTE AND CHRONIC ETHANOL-INDUCED FATTY LIVER

3.1. METHODS

3.2. RESULTS AND DISCUSSION
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>IN VITRO ANTIOXIDANT PROPERTIES OF (+)-CATECHIN COMPOUNDS</td>
<td>150</td>
</tr>
<tr>
<td>4.1</td>
<td>INTRODUCTION</td>
<td>150</td>
</tr>
<tr>
<td>4.2</td>
<td>METHODS</td>
<td>150</td>
</tr>
<tr>
<td>4.3</td>
<td>RESULTS</td>
<td>152</td>
</tr>
<tr>
<td>4.4</td>
<td>DISCUSSION</td>
<td>156</td>
</tr>
<tr>
<td>5</td>
<td>EFFECTS OF (+)-CATECHIN COMPOUNDS ON THE HEPATIC REDOX-STATE AND LIPID UTILISATION AFTER ACUTE ETHANOL ADMINISTRATION</td>
<td>158</td>
</tr>
<tr>
<td>5.1</td>
<td>INTRODUCTION</td>
<td>158</td>
</tr>
<tr>
<td>5.2</td>
<td>METHODS</td>
<td>159</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Animals and Administration of Drugs</td>
<td>159</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Redox-State and Fatty Acid Utilisation studies</td>
<td>159</td>
</tr>
<tr>
<td>5.3</td>
<td>RESULTS</td>
<td>160</td>
</tr>
<tr>
<td>5.4</td>
<td>DISCUSSION</td>
<td>165</td>
</tr>
<tr>
<td>6</td>
<td>MODULATION OF THE REDOX-STATE: INFLUENCE ON ACUTE AND CHRONIC ETHANOL-INDUCED HEPATIC LIPID ACCUMULATION</td>
<td>170</td>
</tr>
<tr>
<td>6.1</td>
<td>INTRODUCTION</td>
<td>170</td>
</tr>
<tr>
<td>6.2</td>
<td>METHODS</td>
<td>172</td>
</tr>
<tr>
<td>6.2.1</td>
<td>Acute in vivo Studies</td>
<td>172</td>
</tr>
<tr>
<td>6.2.2</td>
<td>In vitro Studies</td>
<td>173</td>
</tr>
<tr>
<td>6.2.3</td>
<td>Effect of Methylene Blue on Chronic Alcoholic Fatty Liver</td>
<td>174</td>
</tr>
<tr>
<td>6.3</td>
<td>RESULTS</td>
<td>176</td>
</tr>
<tr>
<td>6.4</td>
<td>DISCUSSION</td>
<td>189</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

EDTA Ethylenediaminotetraacetic acid
PEG Polyethylene Glycol
Tris Tris (hydroxymethylamino) methane
Ci Curie
cpm counts per minute
dpm disintegrations per minute
l litre
v/v volume/volume
w/v weight/volume
g gram
mg milligram : $10^{-3}$ g
µg microgram : $10^{-6}$ g
mol mole
mmol millimole : $10^{-3}$ mol
µmol micromole : $10^{-6}$ mol
nmol nanomole : $10^{-9}$ mol
C.V. Coefficient of Variation
S.D. Standard Deviation
n Number of values
TBA Thiobarbituric Acid
BHT Butylated Hydroxytoluene
DPPD N,N'-Diphenyl-p-phenylenediamine
MB Methylene Blue

The terms ethanol and alcohol are interchangeable in the text of this thesis.
ACKNOWLEDGEMENTS

I am most grateful to my supervisors, Dr J. Chakraborty and Dr A.D. Thomson for the great amount of time and encouragement they gave me during the course of this work.

I am deeply indebted to Dr P. O'Gorman and the staff of the Chemical Pathology Department at Greenwich District Hospital, and to Mr Ken Wraith and his staff at the Animal Laboratory, Bethlem Royal Hospital, for making laboratory facilities available for these studies, and for their valuable assistance at various stages of the laboratory work.

I must thank Zyma (UK), the Medical Council on Alcoholism and the Medical Research Council of Great Britain for financial support for the work, and Zyma (UK) and Du Pont Ltd. for donating some of the drugs used.

I owe thanks to Mrs Jill Wright and Mr Brian Thakeray for carrying out histological examinations in some of the experiments.

Finally, I must thank the people who contributed so much to the final production of this thesis: Mrs Jean Johns for her excellent typing of the manuscript, Mr Peter Kentish for the artwork, and Mr Ray Leng and his staff at the Medical Photography Department, Greenwich District Hospital, for photographic services.
Publications

The following publications arose out of the work presented in this thesis:-


Chapter 1

INTRODUCTION

Many substances have the ability to cause liver injury. These include such widely differing chemicals as carbon tetrachloride, acetoaminophen (paracetamol), retinol (vitamin A), bromobenzene, vinylidene chloride or halothane in combination with hypoxia. In both social and economic terms, probably the most important hepatic toxin of all is ethanol. Although the biochemical effects of this substance on the body are widespread, the consequences of excessive alcohol consumption for the liver nearly always overshadow effects on other sites, although some of its interactions with the functioning of other organs, for example the brain or the pancreas, may prove just as damaging in the long-term. In view of this, the effects of ethanol on the liver have been probably the most studied aspect of this substance's toxicology. Yet, in spite of this, the precise mechanisms, their relative importance, and sequence of occurrence that contribute to the clinical entity referred to as 'alcoholic liver disease' (ALD) are still not completely clear, and often poorly understood. Such difficulties make the task of predicting which substances might be effective hepatoprotective agents a rather haphazard affair, yet over the last few years, development work has yielded some drugs which look promising in terms of limiting tissue damage during ethanol consumption. The aim of this current thesis
was to evaluate the prospective hepatoprotective drug, (+)-catechin, and its palmityl ester, 3-palmitoyl-(+)-catechin, in terms, firstly of establishing these compounds' ability to limit tissue changes following acute or chronic ethanol administration (i.e. chiefly through reducing fatty infiltration or steatosis) and, secondly, to attempt to pinpoint precise biochemical mechanisms by which the substances may be acting to limit steatosis.

1.1 THE PATHOGENESIS OF ALCOHOLIC LIVER DISEASE (ALD)

Clinically, ALD is broken down into three main stages; fatty liver, alcoholic hepatitis and cirrhosis, on the basis of the histological appearance of tissue taken by needle biopsy of the liver. A clearly defined hepatitis stage may not be seen in all patients, such subjects appearing to progress directly from fatty infiltration to the characteristic fibrotic state of cirrhosis. It is often thought that fatty liver is of little consequence as this abnormality disappears after a few weeks of abstinence in the alcoholic. However, it is now becoming evident that whilst fat accumulation is taking place, early fibrotic changes are occurring in the liver lobules which are the precursor lesions of cirrhosis (Lieber, 1983). When considering potential hepatoprotective drugs, substances which prevent steatosis have not traditionally been considered very useful in clinical terms, as this early stage in ALD is thought to be fully reversible. However, in view of the early fibrotic changes now being
seen in association with fatty liver in primates and humans, a drug which can prevent this stage occurring, may also be considerably reducing the risk of the patient developing alcoholic cirrhosis.

1.1.1. Animal Models of Alcoholic Liver Disease

Until the early 1960's it was widely believed that alcohol was not hepatotoxic in its own right, and that alcoholic liver disease was the consequence of the poor nutritional status of alcoholics, who tended to derive nearly all of their calorie intake from ethanol. In the mid-1960's, however, Dr Charles Lieber and his co-workers in New York devised methods of feeding alcohol to experimental animals in so-called 'nutritionally adequate' liquid diets. It was found that administering 36% of the total calorie intake as ethanol to rats in a diet that contained adequate amounts of protein, carbohydrate, vitamins, minerals and lipotropic factors, induced fatty liver after as little as ten days of administration, the severity of the fatty infiltration reaching a peak after about 24 days of alcohol feeding (Lieber et al, 1965; DeCarli and Lieber, 1967). In another study, administration of 36% of the total calories as ethanol to healthy human volunteers, along with an adequate diet, under metabolic ward conditions was also found to induce hepatic steatosis, as confirmed by liver biopsy, suggesting that the rat liquid diet model was relevant to the human situation (Lieber et al, 1965).

The origin of the accumulated fat in the animal studies depended on the experimental design. When the diet contains more than about 20% of the calories as fat, the severity of fatty liver produced is
related to the fat content of the diet, indicating dietary fat as a major source (Lieber and DeCarli, 1970a). In experiments where a fat-free diet is employed, hepatic steatosis is still induced, but here the accumulated fat is presumably derived from endogenous synthesis, with possibly some contribution from adipose tissue fatty acids (Lieber and Spritz, 1966; Mendenhall, 1972).

One of the problems of the rat liquid diet feeding method for alcohol administration is that, although considerable amounts of ethanol are consumed by the animals using this regime (12-15 g/kg/day compared with less than 5 g/kg/day consumed by primates or human alcoholics), steatosis is the most severe lesion that can be produced in this species, even after prolonged ethanol feeding (Lieber and DeCarli, 1982). In order to reproduce lesions comparable to human alcoholic hepatitis and cirrhosis, primate species have to be employed, who will readily consume diets in which 50% of the total calories are derived from ethanol. Methods for feeding alcohol in liquid diets to baboons have been described, a proportion of the animals proceeding, after 1-2 years of feeding, to a full-blown cirrhotic condition (Lieber and DeCarli, 1974; Lieber et al, 1975).

These liquid diet methods of alcohol feeding have clearly demonstrated that ethanol is directly hepatotoxic. However, the possible role of nutrition in human alcoholic liver disease should not be overlooked, as alcoholic subjects are frequently malnourished, and animal work does show that dietary deficiencies alone (eg: of amino acids and choline) can cause liver injury which is further potentiated

One important question to consider in relation to the mechanisms by which ethanol is hepatotoxic, is the relevance of findings made in the rat model to the development of the more advanced lesions seen in primates. Fatty liver has been considered a benign lesion that is fully reversible on abstinence in both experimental animals and humans, so it might be concluded that the mechanisms that produce steatosis are not important in causing hepatitis or cirrhosis. However, there are one or two indications that this may not be the case. Recent studies by Lieber's group (Lieber, 1983) have shown that liver biopsies from alcoholic baboons or patients, that only show steatosis when viewed by light microscopy, do often show some evidence of perivenular fibrosis around the centrilobular hepatic vein when viewed by electron microscopy. It is possible that common mechanisms may be operating to cause both steatosis and this early fibrotic lesion. There is some support for this from the study of Lieber et al (1975), who found higher hepatic triglyceride levels in those experimental baboons at the fatty liver stage, who eventually proceeded to develop cirrhosis. Thus, it may not be inappropriate to consider biochemical mechanisms that cause alcoholic fatty liver in rats as being relevant in the production of alcoholic liver disease in man, and also to consider using agents that protect against fatty liver in rats as treatments for human alcoholic patients.
1.2 BIOCHEMICAL MECHANISMS OF ALCOHOLIC LIVER INJURY

1.2.1. Relationship Between Acute and Chronic Fatty Liver

Many early studies on ethanol hepatotoxicity simply employed large, intoxicating doses of ethanol given to fasted rats (about 6-7 g/kg orally), which caused a transient accumulation of hepatic triglyceride measured 8-24 hours later, returning to control values by 48-72 hours after ethanol dosing (Mallov, 1955; DiLuzio, 1958; Elko et al, 1961). Some workers have considered that the mechanisms underlying this acute alcoholic fatty liver are fundamentally different from those that cause the chronic fatty liver produced in liquid diet feeding studies (Badawy, 1978). Certainly, the origin of the accumulated fat may differ, that after acute ethanol originating from the adipose tissue, whereas after chronic alcohol feeding, the increased hepatic lipid seems to be of dietary origin (Lieber et al, 1966). After chronic ethanol intake, the peripheral fat stores do, in fact, appear to be increased in size (World et al, 1984). However, as will be discussed later, many agents that protect against acute alcoholic fatty liver in rats also prevent chronic ethanol-induced steatosis, suggesting that common mechanisms may be operating to produce both lesions.

1.2.2. The Metabolism of Ethanol: Its Role in Fatty Liver Production

Greater than 90% of the ethanol that is absorbed from the gastrointestinal tract is oxidised in the body, very little being
eliminated unchanged via the kidneys and lungs (Forsander and Raiha-Neils, 1960). Nearly all of this oxidation takes place in the liver, only a small contribution coming from extrahepatic tissues (Larsen, 1959).

The major pathway of ethanol metabolism in the liver involves the cytosolic enzyme alcohol dehydrogenase (ADH), an NAD\(^+\)-dependent dehydrogenase that oxidises ethanol to acetaldehyde. Acetaldehyde is then oxidised to acetate by another NAD\(^+\)-dependent enzyme, aldehyde dehydrogenase (ALDH). The subcellular localisation of the physiologically-important form of the latter has been the subject of much debate. In most species, ALDH activity can be detected in the microsomal, mitochondrial and cytosolic fractions of the cell, although in the rat, it is thought that the mitochondrial ALDH isoenzyme makes the major contribution to \textit{in vivo} acetaldehyde oxidation. In man, one of the cytosolic ALDH isoenzymes is thought to make the greatest contribution to acetaldehyde removal (Parilla et al, 1974; Jenkins and Peters, 1983). Both ADH and ALDH are subject to genetic variation and it has been suggested that lack of a particular ALDH isoenzyme (\textit{ALDH}\(_1\)), may predispose individuals to both an exaggerated response to acute alcohol intake, including facial flushing arising from high circulating levels of acetaldehyde, and possibly also enhance the susceptibility to alcoholic liver disease, again as a result of impaired acetaldehyde removal (Saunders and Williams, 1983; Tipton et al, 1983). Alcoholic patients have been observed to have reduced hepatic aldehyde dehydrogenase activity,
including reduced cytosolic activity of the enzyme, although there has been some argument as to whether this is a primary genetic defect or simply a consequence of alcohol-induced liver damage (Jenkins and Peters, 1980). This defect presumably accounts for the impaired acetaldehyde oxidation that has been reported in alcoholics (Korsten et al., 1975; Schukit and Rayses, 1979; Palmer and Jenkins, 1982), although results from these studies should be viewed with caution as a result of the methodological shortcomings of many acetaldehyde assay methods (Eriksson, 1983). Some workers maintain that this decreased ALDH activity in alcoholics persists with abstinence (Thomas et al., 1982), whereas others have apparently shown that the activity falls during alcohol consumption and reverts to control values on abstinence (Saunders and Williams, 1983; Jenkins et al., 1984).

The end product of ethanol oxidation by these enzymes is acetate. Most of this acetate is utilised extrahepatically, the skeletal muscles extracting the largest part, although acetate is also oxidised in the heart (Williamson et al., 1969; Suokas et al., 1984). Very little ethanol-derived acetate is incorporated into hepatic lipids or oxidised to carbon dioxide in the liver.

The other very important 'by-product' of hepatic ethanol oxidation is hydrogen, manifested in the form of a greatly increased hepatic NADH:NAD ratio (Slater et al., 1964). This hepatic redox-state change resulting from ethanol oxidation may be important in explaining many of the hepatotoxic effects of ethanol, as will be discussed later. The altered redox-state is reflected in the liver
cell as altered metabolite ratios in various redox-pair reactions, such as lactate/pyruvate and 3-hydroxybutyrate/acetoacetate. These ratios can thus be used as indicators of the cytoplasmic and mitochondrial NADH:NAD ratios during ethanol metabolism, both values showing significant increases after acute ethanol dosing (Forsander et al., 1965; Veech et al., 1972). These redox-state changes, apart from being implicated in fatty liver production by a number of reviewers (Sherlock, 1983; Lieber, 1984), may also be responsible for other metabolic derangements induced by ethanol, such as impaired gluconeogenesis and galactose intolerance (Saluspuro and Saluspuro, 1968; Krebs et al., 1969).

Two other pathways by which ethanol can be oxidised as far as acetaldehyde have been described. One involves peroxisomal catalase activity, but is probably of no physiological significance (Hawkins and Kalant, 1972). The other pathway that has received a lot of attention is the microsomal ethanol oxidising system (MEOS). MEOS may contribute something between five and twenty per cent to the overall ethanol elimination rate in vivo in naive animals, and may be of particular importance at high blood ethanol concentrations and after chronic ingestion of ethanol or other enzyme-inducing drugs, when the activity of the system is enhanced. The induction of this MEOS pathway, which involves a specific ethanol-induced form of cytochrome P450 with a high affinity for alcohols and aniline as substrates, may account, in part, for the metabolic tolerance and enhanced ethanol elimination rate observed after chronic ethanol
intake (Lieber and DeCarli, 1968; Lieber and DeCarli, 1970b; Ugarte et al., 1972; Coon et al., 1983). The inducibility of the MEOS pathway and its role in producing metabolic tolerance to ethanol has been neatly demonstrated in a recent study, in which strains of deer mice lacking ADH could be weaned to consume as much ethanol per unit body weight daily as comparable animals with hepatic ADH activity, as a result of MEOS induction (Shigeta et al., 1984). A diagrammatic representation of the various pathways of ethanol metabolism is shown in Fig. 1.1.

A number of studies have indicated that ethanol metabolism is necessary for hepatic lipid accumulation to occur after acute dosing. Morgan and DiLuzio (1970) showed that the fatty liver produced by a single large dose of ethanol (6 g/kg) in rats could be prevented by the ADH inhibitor, pyrazole, arguing against a direct toxic effect of ethanol per se being responsible for the hepatic steatosis. Studies on rat liver slices showed that most of the effects that ethanol exerts on lipid metabolism in this in vitro situation, namely decreased fatty acid oxidation and increased esterification of fatty acids into triglycerides, could be prevented by the addition of 4-methylpyrazole to the system (Blomstrand et al., 1973). Further in vivo studies have shown that ADH inhibitors, including nicotinic acid and sodium diethylbarbiturate, prevent the acute ethanol-induced alterations in hepatic lipid disposal, at the same time as reversing the ethanol-mediated redox-state changes (Rawat and Kuriyama, 1972; Baker et al., 1973).
**FIG. 1.1.** Principal pathways of ethanol and acetaldehyde metabolism in the hepatocyte

**Ethanol**

- NAD → Alcohol dehydrogenase → NADH
- NADPH
- MEOS
- H₂O₂ → Catalase → H₂O
- NADP

**Acetaldehyde**

- NAD → Acetaldehyde dehydrogenase → Acetate
- MEOS: microsomal ethanol-oxidizing system

**Acetate**
The situation during chronic ethanol treatment is not as clear cut. Administration of pyrazole to rats during a chronic liquid diet feeding study in fact enhanced the ethanol-induced steatosis whilst lowering hepatic triglyceride in control animals (Kalant et al., 1972). However, ethanol alone in this study did not cause appreciable hepatic triglyceride accumulation, and pyrazole administration caused marked decreases in dietary intake, body weight and caused some deaths, so that dietary-related effects cannot be ruled out to account for this apparent aggravation of ethanol hepatotoxicity by pyrazole. A more recent study, in which moderate amounts of 4-methylpyrazole were added to alcohol-containing liquid diets, so as to maintain blood ethanol levels at an approximately constant level throughout the day and night, without affecting dietary intake or weight gain, suggested that continuous oxidation of ethanol was an important pathogenetic factor in chronic fatty liver development in the rat, as 4-methylpyrazole enhanced the severity of the ethanol-induced steatosis (Lindros et al., 1983). Thus, it appears that during chronic alcohol intake, as after acute dosing, some consequence of ethanol oxidation is responsible for causing fat accumulation. One possibility is, of course, the increase in the hepatic NADH:NAD ratio and its metabolic consequences. Alternatively, some toxic event mediated by the ethanol metabolite, acetaldehyde, may cause the triglyceride deposition.

1.2.3. Possible Metabolic Consequences of the Hepatic Redox-State Change After Ethanol: Relationship to Fatty Liver Production

The increased NADH:NAD ratio could influence lipid metabolism in a number of ways to favour triglyceride deposition in the liver after
ethanol intake, as follows:

(i) The excess NADH will displace the dihydroxyacetone phosphate: $\alpha$-glycerophosphate redox-pair towards its reduced side, so that hepatic $\alpha$-glycerophosphate levels are increased, thereby favouring esterification of fatty acids into triglycerides (Nikkila and Ojala, 1963).

(ii) Transhydrogenation of reducing equivalents may occur from NADH to NADPH, resulting in an increased NADPH/NADP ratio. There is conflicting data on this matter. Some studies have shown no effect of ethanol on the hepatic NADPH:NADP ratio (Slater, 1964) whereas others have shown a slight increase after ethanol application either in vitro or in vivo (Bucher, 1970; Badawy and Evans, 1981). These discrepancies may be the result of methodological differences between the various studies. However, if ethanol does increase the hepatic NADPH level, this may stimulate fatty acid synthesis through substrate concentration effects on fatty acid synthetase. In vitro work has shown increased incorporation of $^{14}$C-acetate into fatty acids by liver slices in the presence of ethanol (Lieber and Schmid, 1961). This effect was associated with decreased oxidation of either labelled palmitate or acetate to carbon dioxide, suggesting that there was a block in fatty acid oxidation at the tricarboxylic acid cycle stage as a result of ethanol addition. These ethanol-induced abnormalities in lipid metabolism could be reversed by the hydrogen acceptor methylene blue, and were mimicked by sorbitol, another substrate that can induce a redox-state change. This led the authors to propose that it was
the hepatic redox-state changes induced by ethanol that caused the altered hepatic lipid disposal. Reboucas and Isselbacher (1961) however, found that, although ethanol administration to rats did increase incorporation of $^{14}$C-acetate into liver lipids, glucose and sorbitol also achieved this without inducing a fatty liver after acute dosing, and sorbitol caused a similar increase in the hepatic NADH:NAD ratio as that observed after ethanol. Thus, these authors proposed that ethanol did not increase fatty acid synthesis or esterification through a redox-state mediated mechanism. In vitro studies have shown that low concentrations of ethanol (5 mM) do in fact inhibit fatty acid synthesis in isolated hepatocytes (Selmer and Grunnet, 1976). Results from in vivo studies on the effect of ethanol on fatty acid synthesis are also conflicting. In fasted rats, ethanol does not appear to stimulate fatty acid synthesis, whereas in fed rats accelerated lipogenesis has been reported. Chronic ethanol treatment, using a drinking water method of alcohol feeding in rats, which did not induce fatty liver, induced a slight increase in de novo fatty acid synthesis determined using titrated water, although ethanol significantly lowered body weight gain in this study compared with controls, so that dietary factors may account for the differences in this experiment (Cascales et al, 1983). Thus, the possible role of enhanced fatty acid synthesis, secondary to the hepatic redox-state changes in ethanol-induced fatty liver is largely unresolved.

(iii) A third effect effect of ethanol on lipid metabolism has been widely reported, that may be related to the hepatic redox-state
change, and in turn, could be an important factor in the development of fatty liver, this being inhibition of fatty acid oxidation. This has been reported in liver slices (Lieber and Schmid, 1961; Blomstrand et al, 1973; Beauge et al, 1979), isolated hepatocytes (Ontko, 1973) human liver biopsies (Fischel and Oette, 1974) and in vivo. One study in isolated hepatocytes found little effect of ethanol on fatty acid oxidation, although increased fatty acid esterification was found in the same experiments (Wiebe and Belfrage, 1980). Any ethanol-induced decrease in fatty acid oxidation could be the consequence of reduced NAD availability for the various dehydrogenases in the tricarboxylic acid cycle. As mentioned above, Lieber and Schmid (1961) showed that, in vitro, ethanol inhibited oxidation of acetate to carbon dioxide by about the same degree to which it inhibited palmitate oxidation, suggesting the block was at the TCA cycle stage, instead of β-oxidation itself being affected. Another redox-state related effect that could influence fatty acid oxidation is that the NADH generated from ethanol oxidation displaces the NADH normally generated from TCA cycle activity, so that ethanol replaces fatty acids as the source of reducing equivalents for NADH oxidation and oxidative phosphorylation. However, other mechanisms could account for impaired mitochondrial fatty acid oxidation in ethanol intoxication, including a direct toxic effect of acetaldehyde (Cederbaum et al, 1975a). Alternatively, structural alterations that occur as a consequence of long-term ethanol intake seem to impair many mitochondrial functions in their own right, independently of any acute
metabolic effects of ethanol (Cederbaum et al, 1974; Cederbaum and Rubin, 1975).

The inhibition of fatty acid oxidation by ethanol has been proposed as a central effect that could account for ethanol-induced fatty liver, irrespective of the mechanism of this inhibition (Baraona and Lieber, 1979). There is some evidence to support this view. The antihistamine compound chlorcyclizine protects against acute alcoholic fatty liver in rats, apparently reversing the inhibition of palmitate oxidation by ethanol in vivo in the same experiment (Wooles and Weymouth, 1968). Similarly, administration of \( \alpha \)-p-chloro-phenoxy-isobutyrate (Clofibrate) protects against acute ethanol-induced hepatic steatosis in rats, and this hypolipodemic drug is known to cause peroxisome proliferation, and induce peroxisomal fatty acid oxidation which may compensate for the ethanol-induced block in mitochondrial fatty acid oxidation (Brown, 1966). However, the results of this study should be viewed with caution, as clofibrate may also affect peripheral lipolysis. Recent studies using the \( \beta \)-oxidation inhibitor, 4-pentenoic acid have shown that administration of this compound in various dosage regimes causes accumulation of triglyceride in the rat liver, suggesting a link between hepatic fatty acid oxidation rate and triglyceride formation (Thayer, 1984). On the other hand, Reboucas and Isselbacher (1961) concluded in their experiments that depressed fatty acid oxidation was not important in the pathogenesis of ethanol-induced fatty liver, as treatment of ethanol-fed rats with nicotinamide reversed the
inhibition of palmitate oxidation by ethanol but did not prevent the fatty liver. Wooles (1966) also suggested that ethanol-induced fatty liver was not caused by depressed fatty acid oxidation, since, using blood ketone body levels as an index of hepatic fatty acid utilisation, there was no decrease after acute ethanol dosing until near the peak of the triglyceride accumulation. Glucose dosing suppressed blood ketone levels without inducing hepatic lipid accumulation. Wooles (1966) further suggested that ethanol-induced depression of fatty acid oxidation was unrelated to the fatty liver production since Lossow and Chaikoff (1957) had shown glucose dosing depressed $^{14}$CO$_2$ production from l-$^{14}$C palmitate, as had Reboucas and Isselbacher (1961), yet this was not associated with hepatic triglyceride accumulation.

1.2.4. Assessment of Possible Role of Redox-State Related Effects in Hepatic Steatosis.

Firstly, a few comments can be made about the studies quoted above on fatty acid oxidation. The Wooles (1966) study used no direct measure of fatty acid oxidation and failed to take into account any specific effects that ethanol may have on ketogenesis. It has now been demonstrated that acetaldehyde inhibits mitochondrial ketogenesis (Cederbaum et al, 1975). Secondly, effects of ethanol or glucose dosing on peripheral lipolysis have not always been accurately assessed. Many of these early studies used plasma free fatty acid (FFA) levels as indicative of peripheral lipolysis, but this does not take into account flux of fatty acid transport from the periphery to
the liver, the latter organ being very effective at removing unesterified fatty acids from the plasma. Most of the acute ethanol dosing experiments have been carried out in fasted rats, a condition in which peripheral lipolysis and plasma free fatty acid concentrations will be increased. Under these conditions, ethanol appears to have little influence on the rate of peripheral lipolysis or the plasma FFA levels, although some studies demonstrated an increase in the latter after acute ethanol dosing (Brodie et al, 1961; Mallov, 1955). However, glucose dosing depresses peripheral lipolysis under these conditions so that delivery of fatty acids to the liver is decreased, thus any decrease in hepatic fatty acid oxidation under these conditions will not be of any consequence in terms of hepatic triglyceride disposal. Studies in man have in fact shown either no change or a decrease in plasma FFA's during ethanol feeding, and a fall in circulating glycerol (Lieber et al, 1962; Jones et al, 1963; Feinman and Lieber, 1967). Thus, whilst some reviewers have considered increased peripheral lipolysis as an important component of the ethanol-induced fatty liver, it might be more accurate to consider that the inappropriate response of peripheral lipolysis after ethanol ingestion, compared with that after glucose ingestion for example, is a factor necessary for the production of fatty liver after acute ethanol ingestion, rather than this being a mechanism by which the fatty liver arises. Certainly, if peripheral lipolysis is blocked by adrenalectomy or \( \alpha \)-adrenergic blocking agents (Mallov, 1957; Brodie et al, 1963), the severity of
the acute ethanol-induced fatty liver is reduced, but this is probably simply due to lack of fatty acids being supplied to the liver for triglyceride synthesis. Where weights of epidymal fat pads have been used as an index of lipolysis, two studies have found no effect of acute ethanol dosing (Elko et al., 1961; Poggi and DiLuzio, 1965), although at very high doses, alcohol was found to enhance the mobilisation of $^{14}$C-palmitate from prelabelled epididymal fat pads in vivo (Kessler and Yalovsky-Mishkin, 1966) without, however, changing the specific activity of labelling in the hepatic triglycerides.

The role of enhanced peripheral lipolysis in chronic ethanol-induced fatty liver in rats is difficult to assess. One early study showed that chronic ethanol feeding lowered the weights of epididymal and perirenal fat pads, yet no evidence of hepatic steatosis was found in the ethanol-fed animals (Scheig et al., 1966). More recently, the alcoholic fatty liver produced by chronic alcohol feeding has been found to be associated with increased epididymal fat pad weights, suggesting enhanced peripheral lipolysis has little part to play in the hepatic triglyceride accumulation (World et al., 1984), which of course agrees with studies showing that most of the accumulated lipid under these circumstances is of dietary origin (Lieber and DeCarli, 1970a; Mendenhall, 1972).

It will be evident from the above discussion that the effects of acute ethanol ingestion on peripheral lipolysis may be variable and possibly dependent upon experimental conditions. In experiments where oxidation of $^{14}$C-palmitate to $^{14}$CO$_2$ in vivo has been used as an
index of fatty acid oxidation, the effect of ethanol on lipolysis could be important, as this will affect the degree of isotope dilution of labelled palmitate that may take place in the hepatic free fatty acid pool. None of the quoted studies makes any attempt to assess pool sizes after ethanol dosing. Another factor worth bearing in mind is the possible difference in isotope dilution of $^{14}$C-acetate units derived from palmitate in these experiments between ethanol-dosed and glucose-dosed animals. It is possible that acetate units derived from glucose are more effective at diluting the $^{14}$C-acetate pool derived from $^{14}$C-palmitate than are acetate units derived from ethanol metabolism. This may account for the apparent ability of glucose dosing in fasted rats to suppress $^{14}$CO$_2$ production from $^{1-14}$C-palmitate by about the same extent as ethanol. In vitro studies show that ethanol at high concentrations suppresses total carbon dioxide production when baboon liver slices are incubated in the presence of palmitate, by about the same extent as $^{14}$CO$_2$ production from $^{1-14}$C-palmitate falls under the same experimental conditions (Saluspuro et al, 1981). Thus, the specific activity for the evolved $^{14}$CO$_2$ remains approximately constant, suggesting that the acetate derived from ethanol metabolism does not dilute the $^{14}$C-acetyl-CoA pool derived from $^{14}$C-palmitate, but these considerations may not hold true for glucose. Thus, it appears many of the observations of impaired fatty acid oxidation caused by ethanol where $^{14}$CO$_2$ production has been employed, are probably valid, and reflect true inhibition of this mitochondrial function.
One other possible experimental artefact that has to be considered in $^{14}\text{CO}_2$ production studies is the degree of reincorporation of the labelled product into oxaloacetate in the phosphoenolpyruvate carboxylase catalysed reaction. Some studies suggest that as much as one-third of the CO$_2$ generated from Kreb's cycle activity is reincorporated in this fashion, and no information is available on how this may vary in the presence of various substrates, including ethanol and glucose (Tomera et al, 1983). However, taking all the above considerations into account, and also assessing the results of studies where mitochondrial isolated from ethanol-fed animals show decreased ability to oxidise fatty acids in the absence of added ethanol (Cederbaum et al, 1975b; Saluspuuro et al, 1981), there does appear to be a role for this mechanism in the pathogenesis of fatty liver. It is still not clear, however, whether this decrease in fatty acid oxidation is due to the change in the NADH:NAD ratio caused by ethanol metabolism.

Thus, to conclude this section, an effect of ethanol on fatty acid synthesis is somewhat doubtful, although it almost certainly depresses fatty acid oxidation and increases $\alpha$-glycerophosphate levels. Only the last of these effects definitely seems to be a consequence of the hepatic redox-state change after ethanol, and yet this factor may not be important in the pathogenesis of alcoholic fatty liver. Some agents, such as antioxidants, will prevent acute fatty liver without correcting the altered NADH:NAD ratio (Rossiter and Slater, 1973). Antioxidants also prevent the chronic alcoholic
fatty liver (Hartman and Diluzio, 1968). Correction of the hepatic redox-state after acute ethanol administration by tocopheronolactone, did not prevent the development of fatty liver (Hirayama and Hiroshige, 1970). Thus, in spite of the neatness of the redox-state explanation for the production of fatty liver by ethanol, there is evidence to suggest that other factors, that will be discussed below, could be more important in its pathogenesis. A diagrammatic representation of how the ethanol-induced redox-state change may affect lipid metabolism to cause fatty liver is shown in Fig. 1.2.

1.2.5. Role of Acetaldehyde in the Pathogenesis of Alcoholic Fatty Liver

Acetaldehyde can be considered as the 'reactive metabolite' of ethanol metabolism, which would be compared to the quinone-type reactive intermediate derived from paracetamol, which is responsible for the drug's hepatotoxicity. Acetaldehyde reacts readily with amino and sulphydryl groups, forming Schiff base's with lysine residues and reacting covalently with glutathione and glutathione precursors such as cysteine to form hemiacetals. In vitro work has shown acetaldehyde can bind covalently with a number of proteins, including albumin, erythrocyte membrane proteins, hepatic microsomal proteins and hemoglobin (Donohue et al, 1983a; Lumeng et al, 1982; Gaines et al, 1977; Nomura and Lieber, 1981; Stevens et al, 1981; Donohue et al, 1983b). The relevance of this binding to the toxic effects attributed to acetaldehyde is not completely clear, but as will be seen in the ensuing discussion, it may certainly have a role.
Fig. 1.2. POSSIBLE EFFECTS OF THE ETHANOL-INDUCED REDOX-STATE CHANGE ON FATTY ACID METABOLISM
to play in the block in hepatic protein secretion and possibly also
the impairment of mitochondrial functions induced by this ethanol
metabolite. Principally, there seem to be three mechanisms by which
acetaldehyde could contribute to the development of fatty liver.
These are:

(i) Direct inhibition of mitochondrial function
(ii) Inhibition of hepatic protein secretion
(iii) Initiation of lipid peroxidation following ethanol
intake

These will now be considered in turn.

(i) Inhibition of mitochondrial functions by acetaldehyde: In
isolated mitochondria, acetaldehyde inhibits fatty acid oxidation as
assayed by oxygen consumption and CO₂ production. This inhibition is
not mediated by metabolism of acetaldehyde to acetate in the
mitochondria, nor due to impaired translocation of fatty acids into
the organelle. These observations led Cederbaum et al (1975a) and
Cederbaum and Rubin (1975) to suggest that acetaldehyde inhibits
NAD⁺-dependent state 3 oxygen uptake, inhibits oxidative
phosphorylation at site 1, and decreases calcium uptake. These
effects were achieved at added acetaldehyde concentrations of between
0.6 and 12.0 mM in vitro, whereas high concentrations of ethanol,
greater than those that might be achieved in vivo (80 mM) were without
effect. It is pertinent to ask whether these acetaldehyde
concentrations are relevant to possible in vivo effects of the
compound. The hepatic concentration of acetaldehyde during ethanol
oxidation has been reported to be in the range 20-100 μM (Eriksson, 1983; Eriksson et al, 1983), rather lower than the concentrations used in the in vitro studies above. These acetaldehyde concentrations were, however, those added to the incubations, and were not corrected for evaporative losses. These may have been considerable since acetaldehyde boils at 21°C. Acetaldehyde has been shown to be responsible for the in vivo ethanol-induced decrease in phosphorylation potential, caused by increases in ADP and inorganic phosphate levels (Lindros and Stowell, 1982).

These acute effects of acetaldehyde are similar to the functional changes that are observed when mitochondria isolated from rats fed ethanol chronically were compared with those from pair-fed controls. Mitochondria from ethanol-fed rats were much more sensitive to the acute in vitro effects of acetaldehyde than mitochondria from naive rats (Matsuzaki and Lieber, 1977). This suggests that continued exposure to acetaldehyde during chronic alcohol intake causes structural alterations that leads to impaired function, possibly through covalent binding at critical sites on mitochondrial enzyme protein complexes. Ethanol consumption does in fact affect biogenesis of mitochondrial proteins, again an effect that could be mediated by acetaldehyde. For example, the cytochrome a and b contents of mitochondria from ethanol-fed rats is reduced (Rubin et al, 1970). Electron microscopic studies have shown that chronic ethanol feeding profoundly alters mitochondrial morphology, with swelling and abnormal cristae being seen (Rubin and Lieber, 1967;
Rubin and Lieber, 1968). Mitochondria from ethanol-fed rats show altered in vitro calcium transport. Acetaldehyde added in vitro to these preparations at a physiological concentration (100 μM), enhances calcium retention but this effect is abolished by cyanamide (an ALDH inhibitor) pretreatment, indicating that it is acetaldehyde metabolism, rather than acetaldehyde itself that causes this defect (Korsten et al, 1983).

Further studies on mitochondria from ethanol-fed rats showed impaired respiratory control with succinate as the substrate, and loss of control of phospholipase A₂ activity in ageing experiments (Spach et al, 1979). These altered mitochondrial functions were not the result of altered acyl-chain composition of bulk-phase mitochondrial phospholipids, suggesting once again that a direct interaction of acetaldehyde may be responsible. Other studies, however, have shown alterations in the fatty acid composition of mitochondrial phospholipids after chronic ethanol intake (Cunningham et al, 1982).

A number of studies have indicated that the above effects could be mediated through interaction of acetaldehyde with thiol groups. For example, other compounds which are known to interact with thiol groups, such as copper (Cederbaum and Wainio, 1972), and 5,5'-dithionitrobenzoic acid (Hangaard et al, 1969), exert similar inhibitory actions on isolated mitochondria in vitro. Thiol groups participate in many key mitochondrial sites and functions, such as oxidative phosphorylation (Fluharty and Sanadi, 1963; Sabadie-Pialoux
and Gautheron, 1971), and NADH and succinate dehydrogenases (Tyler et al., 1965; Gutman et al., 1972; Slater, 1948). Aldehydes react very readily with mercaptans, and in the case of cysteine, this results in ring closure to form a thiazolidine residue. Indeed, cysteine, at equimolar concentrations to that of acetaldehyde, will protect isolated mitochondria against the inhibition of fatty acid and NAD+—dependent substrate oxidation exerted by this ethanol metabolite. Cysteine also reversed the inhibition of oxygen uptake by acetaldehyde, although only a small effect on the depressed oxidative phosphorylation was observed (Cederbaum and Rubin, 1976).

(ii) **Role of acetaldehyde in ethanol-induced effects on hepatic protein synthesis and secretion:** Two of the earliest and most conspicuous features of ethanol-induced liver damage are fatty infiltration and enlargement of the liver (hepatomegaly). In rats fed ethanol chronically, lipid accumulation only accounts for about half the increase in liver dry weight, the other half being almost totally due to protein retention (Lieber et al., 1965; Baraona et al., 1975). This apparent block in hepatic protein secretion by ethanol was studied further in rat liver slices by Sorrell and Tuma (1978). They found that ethanol alone (10 mM) inhibited both the glycosylation and secretion of prelabelled proteins from liver slice preparations. Low concentrations of acetaldehyde gave similar results. Pulse-chase labelling, or the use of the protein synthesis inhibitors cyclohexamide and puromycin, were employed to differentiate between effects of ethanol and acetaldehyde on protein synthesis and secretion.
Further studies showed that ethanol impaired both the synthesis and secretion of secretory glycoproteins and albumin from rat liver slices (Tuma et al, 1981). Acute studies in isolated hepatocytes showed that ethanol inhibited protein synthesis and secretion (JeeJeeboy et al, 1975; Rothschild et al, 1971), and that this inhibition could be overcome by the addition of agents that corrected the increased NADH:NAD ratios arising in the incubations from ethanol metabolism. Addition of these agents (methylene blue or a combination of asparate and α-ketoglutarate) did in fact increase acetaldehyde concentrations in the incubations, yet still relieved inhibition of protein synthesis by ethanol (Baraona et al, 1980c). However, the relevance of these findings to acute ethanol hepatotoxicity is difficult to assess as there are conflicting reports on the effects of acute ethanol intake on hepatic protein synthesis and secretion in vivo. The study of Baraona et al (1980a) showed no effect of ethanol (3 g/kg p.o.) on hepatic protein synthesis, although reduced secretion of liver protein was reported. Wallin et al (1984) have, however, reported no effect of a similar dose of ethanol (2.88 g/kg p.o.) on either hepatic protein synthesis or secretion. These doses of ethanol do not, however, cause very profound triglyceride accumulation in the liver. Administration of 6 g/kg ethanol orally, a dose that is commonly used to induce acute fatty liver in rats, inhibited glycoprotein and albumin secretion from the liver, this effect being blocked by pretreatment with 4-methylpyrazole, indicating that ethanol metabolism was necessary for the secretory block to occur (Volentine et al, 1984).
More consistent findings have been made in rats after chronic ethanol feeding. Both decreased hepatic protein synthesis and secretion have been reported after chronic ethanol administration to rats (Smith-Kielland et al., 1983; Sorrell et al., 1983). Studies on isolated hepatocytes from ethanol-fed rats apparently showed no decrease in protein synthesis rates when measurements were compared with those in cells from pair-fed control animals. Furthermore, addition of ethanol to these preparations only inhibited protein synthesis in control cells, hepatocytes from ethanol-fed animals being unaffected. This was attributed to the attenuation of ethanol-induced redox-state change in cells from ethanol-fed rats, and these authors proposed that the block in protein synthesis was due to the NADH:NAD ratio increase diverting amino acids away from synthesis to transamination reactions (e.g., alanine to pyruvate), to make up for substrate losses resulting from the redox shift (Baraona et al., 1980a). However, these findings do not agree with effects after chronic ethanol in vivo, suggesting that isolated hepatocytes may be an inadequate system for studying the effects of ethanol on protein synthesis and secretion. One study has shown, that unlike in liver slices and in vivo, ethanol does not impair protein secretion in isolated hepatocytes (Morland et al., 1981).

Studies on the mechanism of impaired hepatic protein secretion certainly indicate that acetaldehyde is implicated. In liver slices, pyrazole prevented the ethanol-induced secretory block, yet correction of the redox-state by methylene blue and pyruvate did not achieve
this. Although sorbitol, which causes redox-state changes, did cause a decrease in secretion in this model, equimolar concentrations of fructose also achieved this, indicating this effect was not related to the redox-state change. Infusion of acetaldehyde alone caused a block in secretion similar to that seen in the presence of ethanol (Sorrell et al, 1977a). This effect of acetaldehyde was irreversible, as washing of the slices after incubation with the substance, and reincubation in fresh, acetaldehyde-free medium, did not reverse the secretory block (Sorrell et al, 1977b). This finding is consistent with a possible role of covalent binding of acetaldehyde to microtubular protein, that could account for the protein accumulation in the liver after chronic ethanol feeding (Baraona et al, 1977; Jennett et al, 1980). Acute ethanol administration has been found to decrease the volume density of microtubules and inhibit tubulin polymerisation in vivo, this effect being exaggerated after long-term ethanol feeding. These effects are further exaggerated if animals have been pretreated with the aldehyde dehydrogenase inhibitor, disulfiram, allowing acetaldehyde to accumulate (Baraona et al, 1979; Baraona et al, 1980b).

Thus, overall, the effects of ethanol on protein synthesis are not completely clear, but there does seem to be a lot of evidence that ethanol consumption impairs hepatic protein secretion, possibly through a covalent interaction of acetaldehyde with microtubules, that in turn may contribute to fatty liver production through hepatic retention of triglyceride in the form of very low density lipoproteins
(VLDL). Although experimental studies show that an increase in plasma triglycerides (TG) is associated with the development of fatty liver in man (Lieber et al., 1965; Allen and Adena, 1985) there is evidence that this may be a transient phenomenon, and that after continued long-term intake, plasma TG levels do in fact fall, supporting the hypothesis that hepatic retention of VLDL could contribute to fatty liver formation (Belfrage et al., 1977).

(iii) Acetaldehyde: its role in ethanol-induced lipid peroxidation:
There is experimental evidence to indicate that acetaldehyde is responsible for initiating lipid peroxidation after acute ethanol exposure. In view of the controversial nature of lipid peroxidation in ethanol-induced liver injury, and its relevance to the work described in this thesis, this aspect is fully discussed in the forthcoming section.

1.2.6. Ethanol-Induced Hepatic Lipid Peroxidation

(i) The role of lipid peroxidation in ethanol-induced liver injury:
The role of lipid peroxidation in ethanol-induced liver injury has been the subject of much debate over the years. For certain hepatotoxic compounds, notably carbon tetrachloride, it is widely accepted that the initial event involved in its hepatotoxicity is metabolism to a free radical intermediate, which can then interact with a number of neighbouring macromolecules in the cell, including covalent binding and peroxidation of unsaturated fatty acid residues of membrane phospholipids. The precise link between these early free radical-mediated reactions, and the ultimate breakdown of hepatocyte
function and viability in carbon tetrachloride poisoning is not understood, but lipid peroxidation is thought to play a part in this process (Brattin et al., 1985). The initial hepatic change after carbon tetrachloride administration in experimental animals is similar to that after ethanol, namely an increase in hepatic triglycerides.

The concept that lipid peroxidation might be responsible for the ethanol-induced fatty liver in rats was first proposed by Diluzio (1964). He showed that the acute ethanol-induced fatty liver could be prevented by the co-administration of an antioxidant cocktail (G-50, which contains butylated hydroxytoluene, butylated hydroxyanisole, and propyl gallate). Further studies showed that the synthetic antioxidant N,N'-diphenyl-p-phenylenediamine (DPPD) and alpha-tocopherol acetate (vitamin E) also protect against acute ethanol-induced steatosis in rats (Diluzio, 1966). Some support for a role of lipid peroxidation in the ethanol-induced fatty liver came from a study in which DPPD, in an identical dosage regime to that which protected against ethanol-induced liver injury, also prevented acute carbon tetrachloride hepatotoxicity (Diluzio and Costales, 1965). About this time, there was also much speculation as to the possible role of the altered NADH:NAD ratio after ethanol dosing in the production of fatty liver, so that some workers, including Diluzio (Diluzio, 1965; Diluzio and Costales, 1965), proposed that antioxidants might protect against steatosis through modulation of ethanol-induced redox-state change. This question was not fully resolved until Rossiter and Slater (1973) clearly showed that a
variety of antioxidants (DPPD, BHT, and propyl gallate) prevented the acute alcoholic fatty liver without reversing the increased NADH:NAD ratio. This suggested that antioxidants may indeed be acting through inhibition of lipid peroxidation after ethanol dosing.

Much of the controversy over the role of lipid peroxidation in alcoholic liver damage centres on the evidence that has been produced in an attempt to demonstrate that ethanol intake causes increased peroxidation of hepatic lipids. Using an iodometric technique, Kalish and DiLuzio (1966) demonstrated increased liver lipid peroxide levels after acute ethanol dosing, which were prevented by antioxidant administration (co-enzyme Q4), and at the same time the latter substance prevented fatty liver. DiLuzio and Hartman (1967) also reported increased liver malondialdehyde content after acute ethanol dosing in rats, which, again, was prevented by antioxidant pre-treatments, in the same experiments in which antioxidants inhibited steatosis. Furthermore, treatment of animals with DPPD during chronic feeding of ethanol as 36% of the calories intake in a liquid diet, also prevented the chronic ethanol-induced fatty liver (Hartman and DiLuzio, 1968). These findings led DiLuzio to propose lipid peroxidation as an important mechanism of ethanol-induced liver injury (DiLuzio, 1973).

The controversy relating to these views arose in the latter part of the 1960's. Firstly, Lieber and DeCarli (1966) found that addition of either DPPD or \( \alpha \)-tocopherol acetate to their alcohol-containing liquid diets did not prevent fatty liver after
chronic ethanol administration. This may, however, be due to poor intestinal absorption of lipid soluble antioxidants such as DPPD. It is now recognised that to be effective, an antioxidant has to be in the right place, at the right time, and at a suitable concentration (Slater, 1984) and this may not have been achieved in this study. There was also some dispute as to whether acute ethanol dosing induced hepatic lipid peroxidation in the rat. Hashimoto and Recknagel (1968), using the sensitive and specific diene conjugate assay for detecting lipid peroxidation, found no evidence of peroxidation in any of the subcellular liver fractions studied (microsomes, mitochondria or nuclei) after a large, acute oral dose of ethanol (7.9 g/kg body weight), whereas low doses of carbon tetrachloride produced clear evidence of diene absorption in the microsomal lipids using identical methodology. These authors also failed to find any evidence of increased liver lipid peroxides, measured iodometrically, after the same ethanol dose. DiLuzio replied to these studies (DiLuzio, 1968), producing data that did in fact show some evidence of diene conjugation in the mitochondrial lipids only, one hour after a slightly lower dose of ethanol (6 g/kg). DiLuzio criticised Hashimoto and Recknagel's iodometric technique for determining liver lipid peroxides, and reported a 100% increase in liver lipid peroxides measured six hours after ethanol dosing, although there was no significant increase at earlier time points (DiLuzio, 1968). Thus, it appears that the discrepancies between the Hashimoto and Recknagel and DiLuzio's data may arise from differences in methodology, the
ethanol doses employed, and the time points studied. The very large dose used by the former authors is close to the LD$_{50}$ dose for ethanol in rats, and this dose might be expected to cause inhibition of ethanol metabolism due to the high liver ethanol concentrations that could be achieved. Alcohol dehydrogenase activity is decreased at high ethanol concentrations, and more recent studies have shown that ethanol metabolism is necessary for ethanol-induced lipid peroxidation to occur in vivo (Bosron et al., 1983; Sippel, 1983). In addition, ethanol itself is an effective radical scavenger, so that at these high ethanol levels, any peroxidative processes may in fact be counteracted (Videla and Valenzuela, 1982).

However, some other workers questioned the role of lipid peroxidation in ethanol hepatotoxicity. Bunyan et al. (1969) failed to find any increase in liver lipid peroxides, measured iodometrically, after acute ethanol dosing in rats (5-8 g/kg orally), although they did find that ethanol added to liver homogenates in vitro increased malondialdehyde production. These authors also confirmed DiLuzio's finding that the DPPD prevented the ethanol-induced fatty liver, although vitamin E was not effective in this respect.

Since the controversy arose over ethanol-induced lipid peroxidation and its role in hepatotoxicity, a number of studies have been published that do show that ethanol induces lipid peroxidation, although it is clear that it is a much weaker pro-oxidant than carbon tetrachloride. Comporti et al. (1973) showed production of
malondialdehyde is increased in liver homogenates taken from rats after acute ethanol administration, and that the reduced glutathione content of liver was decreased, possibly as a result of increased peroxidation. Macdonald (1973), using a diene conjugation method for detecting lipid peroxidation, found increases in the liver mitochondrial lipids after acute ethanol administration (5 g/kg orally), and also found that susceptibility to peroxidation in both the mitochondrial and microsomal lipids was enhanced by repeated doses of ethanol over a two week period. This author also found increases in liver glutathione peroxidase and glutathione reductase activities after long-term dosing with ethanol. No liver lipid measurements were made in this study however. Sippel (1983) found increased diene conjugates in liver microsomal lipids at an early time point (2 hours) after a moderate intraperitoneal dose of ethanol (1.5 g/kg), this increase occurring in the absence of any fall in hepatic reduced glutathione levels. Using a newly-developed second derivative spectroscopic method for detecting diene conjugation, Corongiu et al (1983) reported microsomal lipid peroxidation 24 hours after acute ethanol administration (7.9 g/kg orally). Valenzuela et al (1980) found increased diene conjugation in total liver lipids and increased superoxide dismutase and NADPH oxidase activities in liver homogenates taken from rats 6 hours after acute ethanol administration (5 g/kg orally).

In the isolated perfused rat liver, Muller and Sies (1982) found low levels of ethanol (0.5 mM) in the perfusate increased ethane and
pentane production by the organ, this increase being blocked if pyrazole or 4-methylpyrazole was added to the system. Increased alkane production could also be initiated by adding acetaldehyde to the perfusate in the presence of pyrazole, indicating that ethanol itself, or possibly the presence of ethanol at a cytochrome P450 binding site, is not the initiating factor for peroxidation to occur. The data suggests that it is some acetaldehyde-mediated action that is critical in this respect.

Rouach et al (1983) have also reported hepatic lipid peroxidation (measurement of thiobarbituric acid reactive material, thought principally to be malondialdehyde, MDA) during ethanol administration using an inhalation method, mitochondria being mainly affected. In accord with the study of Macdonald (1973), they found that susceptibility to peroxidation was enhanced after four days of ethanol inhalation.

Observations on the occurrence of, and the role of, lipid peroxidation during chronic ethanol feeding in experimental animals is rather limited. As already mentioned, repeated ethanol dosing appears to enhance the susceptibility of liver lipids to the peroxidation induced by an acute dose of ethanol (Macdonald, 1973). A study by Torrelli et al (1978) failed to demonstrate any evidence of microsomal lipid peroxidation measured as diene conjugation after acute ethanol administration (7 g/kg orally) or after up to three weeks administration of a daily ethanol dose of 3 g/kg orally. However, production of TBA-reactive substances by liver homogenates
from animals in this study was increased by the ethanol treatments. This ethanol dosage regime would not normally be considered a suitable method for the chronic administration of ethanol, and the study of Shaw et al (1981) is probably the most comprehensive in terms of the effect of a chronic ethanol feeding regime (using the Lieber-DeCarli liquid diet) on hepatic lipid peroxidation. This study could find no significant difference in microsomal or mitochondrial diene conjugates in rats after six weeks of ethanol feeding, but administration of a moderate dose of ethanol (3 g/kg) to ethanol-fed animals caused marked increases in diene conjugates in both subcellular fractions, but had little effect in pair-fed control animals. There was no hepatic glutathione depletion associated with this ethanol dose in either control or ethanol-fed animals. One of the most interesting aspects of this study, was however, the data obtained from baboons which had been fed ethanol in liquid diets for up to four years. There was a significant increase in diene conjugates in total liver lipids, and depressed liver glutathione levels in ethanol-fed animals when compared with controls, without having to give an acute ethanol challenge as in rats. Furthermore, administration of a moderate dose of ethanol (1.6 g/kg) caused marked hepatic glutathione depletion and increases in hepatic diene conjugates, these changes being greatest in the ethanol-fed animals, again highlighting the increased susceptibility to lipid peroxidation after chronic alcohol intake. Thus, it appears that primates are much more susceptible to ethanol-induced lipid peroxidation than rats, which is of interest,
as, of course, primates develop the whole spectrum of hepatic lesions seen in man during long-term alcohol intake, whereas rats only proceed to the fatty liver stage. This suggests a possible causal link between hepatic lipid peroxidation and ethanol hepatotoxicity.

Studies in human alcoholics have shown increased liver lipid diene conjugates and depressed reduced glutathione levels in patients with alcoholic liver disease, when compared with patients with liver disease of non-alcoholic origin (Shaw et al, 1983).

(ii) Possible mechanisms of ethanol-induced lipid peroxidation: The perfused liver study of Muller and Sies (1982) and the in vivo study of Sippel (1983), as mentioned above, indicate that in the rat, ethanol metabolism is a requirement for lipid peroxidation to occur after acute exposure. This, once again, incriminates the ethanol metabolite, acetaldehyde, in the process. There are a number of ways in which acetaldehyde could cause lipid peroxidation. Firstly, its ability to interact with thiol groups, could cause depletion of reduced glutathione or glutathione precursors such as cysteine, thus increasing endogenous peroxidation. Both acute and chronic ethanol treatments cause hepatic glutathione depletion in rats and baboons (Comporti et al, 1983; Macdonald et al, 1977; Shaw et al, 1981; Videla et al, 1980; Fernandez and Videla, 1981). In vitro studies show that pyrazole abolishes the decrease in hepatic GSH induced by 20mM ethanol, whereas the ALDH inhibitor, disulfiram, potentiates it, suggesting a role for acetaldehyde (Vina et al, 1980). After chronic ethanol, increased glutathione breakdown may be an important
mechanism, as the activity of hepatic \( \gamma \)-glutamyl transpeptidase is increased after long-term ethanol intake (Teschke et al, 1977; Ishii et al, 1978). However, some studies show that ethanol will cause lipid peroxidation in the absence of glutathione depletion (eg: Sippel, 1983), so mechanisms other than simply loss of reduced glutathione may be involved.

A number of enzymatic mechanisms have been proposed by which acetaldehyde and/or ethanol could cause lipid peroxidation. Acetaldehyde can act as a substrate for both aldehyde oxidase and xanthine oxidase, both enzymes yielding superoxide anions when they oxidise substrates. Xanthine oxidase normally exists \textit{in vivo} as an NAD-dependent dehydrogenase that catalyses the formation of uric acid from xanthine. Under certain adverse conditions, (eg: hypoxia), the enzyme can lose a critical thiol group, and become converted to the superoxide-producing form (Type 0) of the enzyme, referred to as xanthine oxidase (Stirpe and Della Corte, 1969; Della Corte and Stirpe, 1972). This interconversion, yielding copious amounts of superoxide on reperfusion of a tissue with oxygenated blood after an ischaemic episode, has been proposed as the mechanism by which tissue damage may occur in ischaemic heart disease (McCord, 1985). During ethanol metabolism, the increased oxygen consumption and centrilobular hypoxia that can occur in the liver might favour conversion of xanthine dehydrogenase into the oxidase form (Baraona et al, 1983; Thurman et al, 1984). It has been proposed that such an interconversion, and the subsequent oxidation of ethanol-derived
acetaldehyde to yield superoxide anions, could underly ethanol hepatotoxicity (Lewis and Paton, 1982).

Chronic ethanol treatment does cause an increase in the liver xanthine oxidase:xanthine dehydrogenase ratio (Oei et al, 1982), although comprehensive studies on the effect of a xanthine oxidase inhibitor (e.g., allopurinol) on ethanol hepatotoxicity are required to assess the role of this mechanism. However, it does seem likely that some enzymatic process is involved in ethanol-induced lipid peroxidation, the latter then being exacerbated by the glutathione depletion. As already mentioned, loss of glutathione is probably not the sole explanation for ethanol-induced peroxidation, as the lowering of glutathione levels with diethyl maleate is not associated with increased lipid peroxidation in the liver (Wendel et al, 1979; Lindstrom and Anders, 1978).

Enzymatic processes are probably also important in the enhanced susceptibility to peroxidation after long-term ethanol intake. Proliferation of the smooth endoplasmic reticulum and increases in associated enzyme activities, such as NADPH oxidase, ethanol-induced forms of cytochrome P450 and cytochrome-c reductase, could enhance superoxide production (Reitz, 1975; Boveris et al, 1983). This increased capacity of the microsomal fraction to produce superoxide anions after chronic ethanol treatment, in the absence of a parallel increase in cytosolic superoxide dismutase activity, could underly the enhanced susceptibility to peroxidation (Boveris et al, 1983). Superoxide anions in their own right are not very reactive or
damaging, conversion to the highly-reactive hydroxyl radical, by means of iron-catalysed Fenton-type reactions, often being necessary before significant, potentially cell-damaging, events occur (Freeman and Crapo, 1982; Slater, 1984). Microsomal generation of hydroxyl radicals is enhanced after chronic ethanol treatment, but this increase does not correlate precisely with endogenous microsomal lipid peroxidation measured in vitro (Shaw et al, 1984).

In conclusion, it will be clear that the role of lipid peroxidation in ethanol-induced liver injury, and the underlying mechanisms are still the subject of much debate. It does appear, however, that some enzymatic metabolism of acetaldehyde in combination with glutathione depletion, initiated by this ethanol metabolite, may increase lipid peroxidation in the liver, and that after chronic treatment, microsomal free radical production may be increased, so as to enhance susceptibility to lipid peroxidation. The protective effects of antioxidants on fatty liver in rats, the greater susceptibility of primates to peroxidation, and the increased severity of hepatic lesions seen after ethanol intake in the latter species, all suggest a role for lipid peroxidation in ethanol-induced liver injury. The link between ethanol-induced lipid peroxidation and some of the other impaired hepatic functions that have been implicated in ethanol hepatotoxicity (eg: mitochondrial damage/dysfunction, microtubule damage and impaired glycoprotein secretion), remains to be established, but investigations of such possible inter-relationships could prove fruitful in clarifying the biochemical mechanisms of ethanol-induced liver damage.
1.3. OTHER POSSIBLE MECHANISMS OF FATTY LIVER PRODUCTION BY ETHANOL

1.3.1. Role of ethanol-derived acetate

The copious amounts of acetate produced by hepatic ethanol oxidation could act as a precursor of the accumulated hepatic lipids under such conditions. However, most of this acetate is oxidised extrahepatically, chiefly in the heart and skeletal muscle (Fellenious et al, 1973; Damgaard et al, 1973). One study did find that administration of acetate to rats caused a similar degree of triglyceride accumulation, and a redox-state shift almost identical to that when an equivalent dose of ethanol was given (Morgan and Mendenhall, 1977). These authors proposed that ethanol-derived acetate displaced fatty-acyl-CoA derivatives as substrates for mitochondrial oxidation, thereby causing triglyceride accumulation. They also proposed that acetate oxidation contributed to the redox-state change after ethanol rather than the excess NADH being derived solely from oxidation of ethanol to acetate. The possibility, however, that the steatotic effect of acetate was mediated by acetaldehyde formed from acetate, through reduction by aldehyde dehydrogenase was not investigated. Acetate has also been shown to inhibit peripheral lipolysis and cause a fall in circulating free fatty acids, effects which might in fact be thought to counteract the production of a fatty liver (Nilson and Belfrage, 1978; Crouse et al, 1968). Thus, a role for ethanol-derived acetate in the pathogenesis of hepatic steatosis seems doubtful.
1.3.2. Increased peripheral lipolysis

Early studies in rats (Brodie et al., 1961; Elko et al., 1961) suggested that increased mobilisation of fatty acids from peripheral depots could account for alcoholic fatty liver. Studies in man did not agree with this concept however (Crouse et al., 1968). In view of the more detailed discussion earlier in this chapter (Section 1.2.4), and the finding in rats that epididymal fat pads weights are increased after chronic ethanol feeding (World et al., 1984), it is doubtful whether increased peripheral lipolysis is an important primary mechanism by which ethanol causes fatty liver.

1.3.3. Induction of Specific Enzyme Activities

Acute ethanol ingestion increases the activity of hepatic phosphatidate phosphohydrolase, the rate-limiting enzyme in triglyceride synthesis (Savoleinen, 1977; Pritchard et al., 1977), increases also being seen after chronic ethanol treatment (Lamb et al., 1979). The increase in this activity precedes the ethanol-induced triglyceride accumulation. The cause of induction appears to be increased serum corticosterone concentrations arising in response to a large intoxicating dose of ethanol, adrenalectomy largely preventing the ethanol-induced increase in phosphohydrolase activity (Brindley et al., 1979). However, ethanol-induced stimulation of phosphatidate phosphohydrolase activity in the soluble fraction of liver is probably due to activation of an existing phosphohydrolase, partly by glucocorticoids and partly by α-glycerophosphate (Savoleinen and Hassinen, 1978), rather than true induction taking place (Sturton et al., 1981).
More recent work indicates that phosphatidate phosphohydrolase normally exists in latent form in the soluble fraction of the liver, but undergoes activation and translocation to the microsomal fraction (Cascales et al., 1984). This process is promoted by unesterified fatty acids. It is therefore possible that the enhanced activity seen after ethanol, prior to triglyceride accumulation in the liver, is, in part, a response to increased free fatty acid availability in the liver secondary to some other ethanol and/or acetaldehyde-mediated event (e.g., inhibition of fatty acid oxidation). The hepatic concentration of free fatty acids is increased in patients with alcoholic liver disease (Mavrelis et al., 1983).

The first enzyme in the pathway of hepatic glycerolipid synthesis, sn-glycerol-3-phosphate acyltransferase, is induced by chronic ethanol feeding, a 73% increase being observed after six weeks in rats (Joly et al., 1973). Diacylglycerol acyl transferase, the last enzyme in the pathway of triglyceride synthesis, and the only enzyme specific for triglyceride formation in the pathway (the others are common to phospholipid synthesis), is another enzyme that could be affected by ethanol treatment, since most of the accumulated lipid is triglyceride. One study showed no effect of acute ethanol treatment on the activity of this enzyme (Pritchard et al., 1977). However, the activity of this enzyme is controlled by a phosphorylation-dephosphorylation mechanism (Haagsman et al., 1982), so that when the cytosolic fraction is included in the assay for activity in microsomes, a two-fold increase can be observed after ethanol
dosing (Vaaninen et al., 1981). Increases in other microsomal enzymes that may be important in ethanol-induced lipid peroxidation have already been discussed. The enzyme induction that has been described here could certainly explain the distribution of accumulated fat between the lipid classes that occurs after ethanol intake, although it seems likely that these changes, in particular the effects on phosphatidate phosphohydrolase, occur in response to other effects of ethanol on fatty acid metabolism, rather than enzyme induction being a primary mechanism by which triglyceride deposition occurs.

1.3.4. Increased Hepatic Blood Flow

Abrams and Cooper (1976a) carried out a quantitative analysis of the hepatic triglyceride accumulation following two moderate acute doses of ethanol (2.1 g/kg and 0.7 g/kg i.p.). At the higher dose, half the accumulated hepatic triglyceride could be accounted for by increased hepatic uptake of unesterified fatty acids from the serum. At the lower dose, this source could account for all of the accumulated triglyceride. They went on to show that this increased uptake of fatty acids correlated with the increase in the serum volume of the liver, and the increase in hepatic blood flow (measured using a radiolabelled colloid) after the same ethanol dose (Abrams and Cooper, 1976b). These authors proposed that increased hepatic blood flow, with a concomittent increase in hepatic uptake of fatty acids is responsible for the fatty liver after this low ethanol dose, since the rate of triglyceride formation is related to fatty acid availability in the liver, and there was no increase in lipoprotein secretion after
ethanol to counteract the increased triglyceride synthesis.

A number of points can be made about these studies. Firstly, this mechanism only accounts for the mild degree of fat accumulation after the 0.7 g/kg ethanol dose, the high dose (2.1 g/kg) causing a more pronounced rise in hepatic triglycerides that could not be accounted for solely by increased serum uptake of fatty acids. The low dose of ethanol does not produce the classic, histologically observable, centrilobular fatty infiltration caused by larger oral doses of ethanol (5 g/kg) or chronic ethanol feeding in liquid diets, when 12-15 g/kg/day ethanol may be consumed. Furthermore, chronic ethanol treatment causes an increase in liver size, due to hepatocyte enlargement, that in fact reduces hepatic blood flow, yet fatty liver persists in this condition (Israel and Orrego, 1983). Thus, increased hepatic blood flow is probably only important in causing fat accumulation after low doses of ethanol, and makes very little contribution to the more severe, and pathologically important, steatosis that occurs after large acute doses of ethanol or long-term alcohol intake.

1.3.5. Hypoxia and Disturbances in Thyroid Hormone Metabolism

The clearance of ethanol from the blood of animals and humans is enhanced after chronic ethanol intake, due to increased hepatic metabolism of ethanol (Lieber, 1973; Kater et al., 1969; Pieper and Skeen, 1973; Tobon and Mezey, 1971). This increase coexists with a generalised 'hypermetabolic state' after chronic ethanol ingestion, which is demonstrated by enhanced oxygen requirements. Increased
oxygen consumption occurs in rats fed alcohol chronically and human chronic alcoholics (Pirola and Lieber, 1975; Pirola and Lieber, 1976). The mechanism underlying this increased consumption is disputed. Since it can be inhibited by oubain, Bernstein et al (1973) suggested it was a consequence of enhanced Na\(^+\)-K\(^+\)-ATPase activity causing ATP consumption, thereby increasing mitochondrial oxygen demands for electron transport and oxidative phosphorylation. By this mechanism, NADH reoxidation would be enhanced, thus possibly accounting for the attenuation of ethanol-induced hepatic redox-state changes after chronic ethanol intake (Teschke et al, 1973), and also explaining the increased ethanol metabolism, as NADH reoxidation has been considered a rate-limiting step in ethanol oxidation (Badawy, 1978). However, pyrazole treatment does not completely prevent the enhanced ethanol elimination rate after chronic intake, so alternative mechanisms may be important. Indeed, the microsomal ethanol oxidising system, described earlier, may account for this phenomenon (Cederbaum et al, 1978). In one study, an enhanced ethanol elimination rate was observed in isolated, perfused rat livers and cultured hepatocytes taken from ethanol-fed rats without a concomittant increase in oxygen consumption (Schaffer et al, 1980). Yuki et al (1980) reported that the influence of oubain on ethanol metabolism was non-specific and need not implicate Na\(^+\)-K\(^+\) ATPase activity. Gordon (1977) reported lowered hepatic ATP levels in rats after long-term ethanol intake, suggesting that the injured mitochondria were unable to adapt to produce more ATP under these
circumstances.

Whatever the underlying mechanisms, it is apparent that ethanol metabolism accentuates the oxygen gradient across the liver lobule from the arterial to the venous side, a centrilobular hypoxia occurring. This has been demonstrated in some elegant experiments with miniature oxygen electrodes performed by the group of Thurman (Ji et al, 1982; Matsumara and Thurman, 1983). Experiments with perfused livers have shown that anoxic stress led rapidly to centrilobular injury with sparing of periportal areas. This could indicate that centrilobular hypoxia might be important in the pathogenesis of alcoholic liver disease.

The reason for implicating thyroid hormones in the above hypotheses is that treatment of animals with thyroid hormones produces similar changes in oxygen consumption and Na\(^+\)-K\(^+\)-ATPase activity in the liver as those caused by ethanol (Israel et al, 1973). Thus, it appears that a hypermetabolic state, akin to that in hyperthyroidism, may exist in the livers of alcohol-fed animals and humans. However, there is little evidence to suggest that alcohol consumption brings this situation about through interference in thyroid hormone metabolism. Although many of the changes that occur in the liver of hyperthyroid animals (eg: megamitochondria, proliferation of the smooth endoplasmic reticulum) are similar to those seen after ethanol (Israel et al, 1973), serum thyroid hormone levels seem to indicate that patients with alcoholic hepatitis are euthyroid, or if anything, slightly hypothyroid (Chopra et al, 1974; Israel et al, 1979).
Nevertheless, the 'intracellular hyperthyroidism' or 'hypermetabolic state' theory of alcoholic liver disease and its possible role in causing centrilobular hypoxia and necrosis, has remained an attractive one for many workers. This led to clinical trials of the antithyroid drug, propylthiouracil, in the treatment of alcoholic hepatitis. Animal studies showed that propylthiouracil protects rats which had been fed ethanol chronically from hypoxia-induced centrilobular necrosis (Israel et al, 1975). Clinical trials of the drug in patients with alcoholic hepatitis have shown either no benefit (Halle et al, 1982) or only marginal improvements in outcome, mortality being unaffected in both of these studies (Orrego et al, 1979). A number of criticisms have been levelled at these trials, including the small numbers of patients employed and inadequate statistical methods (Maddrey, 1979; Kaplowitz, 1982).

With regard to the mechanism of these effects, propylthiouracil will also protect animals against carbon tetrachloride and acetoaminophen (paracetamol) hepatotoxicity (Linscheer et al, 1980; Orrego et al, 1976). These effects appear to be independent of any antithyroid action of the drug. In the case of paracetamol, it has been shown that the drug increases hepatic reduced glutathione levels (Linscheer et al, 1980), and can act as a substitute for glutathione to form a stable adduct with the active metabolite of acetoaminophen, thereby preventing its toxicity (Yamada et al, 1981). Thus, for ethanol, propylthiouracil may act non-specifically through inhibiting lipid peroxidation, as a result of increased hepatic glutathione
levels, or it may form an adduct with acetaldehyde in a manner similar to that reported for the thiol compound, cysteine, thus protecting against the toxic effects of this ethanol metabolite (Hirayama et al, 1983).

Therefore, there is still some dispute about whether the 'hypermetabolic state' or hypoxia exists in alcoholic liver disease (Thurman et al, 1984), and whether it is important in the pathogenesis of this condition. If centrilobular hypoxia does occur in human alcoholics, as is suggested by animal studies, it may trigger another mechanism of damage that has already been discussed, that is free radical generation and lipid peroxidation, tissue damage occurring in a manner similar to that of ischaemic heart disease (McCord, 1985). As to the role of thyroid hormones in the hypermetabolic state theory of alcoholic liver disease, their implication seems unlikely in view of the effects of propylthiouracil observed in relation to acetaminophen hepatotoxicity (Yamada and Kaplowitz, 1983; Yamada et al, 1981). Further studies on the mechanisms involved are need to clarify this point (Szilagyi et al, 1982).

1.3.6. **Disturbances in Essential Fatty Acid and Prostaglandin Metabolism**

Chronic ethanol intake in experimental animals changes the fatty acid composition of the hepatic phospholipids. In particular, increased linoleic acid (18:2) and decreased arachidonic acid (20:4) contents are found, so that the 20:4/18:2 ratio is decreased, particularly in the mitochondrial fraction of the liver, although the
microsomes are also affected (Reitz, 1975; Thompson and Reitz, 1978; Schilling and Reitz, 1980; Cunningham et al, 1982; French et al, 1971). Other polyenoic fatty acids have also been observed to decrease. These changes could be important in determining membrane fluidity and membrane-associated functions after chronic ethanol, that, in turn could affect functioning of hepatocyte organelles (eg: mitochondria), so that changes in lipid disposal occur that could produce fatty liver.

One explanation for the changed fatty acid profiles might be ethanol-induced lipid peroxidation (Reitz, 1975). The reactivity of free radical species with unsaturated fatty acids increases with the number of double bonds present, and in view of the high proportion of phospholipid acyl groups present as arachidonic acid in liver, this fatty acid might be a particularly susceptible target for free radical attack (Slater, 1981). This mechanism could account for the loss of arachidonic acid and the decreased double bond index/saturated fatty acid ratio seen in animals and humans after ethanol intake (Reitz, 1975; Cairns and Peters, 1983). However, this mechanism has not been proven and awaits experiments in which the influence of antioxidant treatment on ethanol-induced changes of fatty acid composition is determined, in order to achieve acceptance.

More recently, there has been much interest in the role of altered microsomal fatty acid desaturase activities after ethanol treatment in causing fatty acid composition changes. The essential fatty acids of the n-6 series are probably the most important in
animal tissues, although the n-3 series (derived from alpha-linolenic acid) may be important in the brain and cardiovascular system. Linoleic acid (18:2) is the most abundant dietary essential fatty acid and is important as the precursor for the n-6 series of fatty acids, including arachidonic acid. Conversion of linoleic acid to arachidonic acid involves two desaturation steps, catalysed by microsomal Δ-6-desaturase and Δ-5-desaturase enzymes, and also a microsomal elongase. The activities of cytochrome b₅-dependent Δ-5, Δ-6 and the Δ-9 desaturases are all decreased in rat liver microsomes after chronic ethanol feeding (Wang and Reitz, 1983; Umeki et al, 1984). This mechanism may therefore explain the changes in fatty acid composition. In addition, ethanol has been shown to increase the conversion of dihomogamma-linolenic acid (20:3w6) to prostaglandin El, thereby depleting this acid as a source of arachidonate in membrane phospholipids (Manku et al, 1979; Rotrosen et al, 1980).

These effects of ethanol on essential fatty acids and prostaglandins have been proposed as a mechanism by which tissue damage, and some of the behavioural consequences of chronic ethanol consumption arise (Horrobin, 1980). There is some experimental evidence to support this. Supplementation of alcohol-containing liquid diets with arachidonic acid prevents the formation of fatty liver (Goheen et al, 1980; Goheen et al, 1983; Karpe et al, 1984), as well as preventing the fatty liver arising from a high-fat diet. However, in some studies, arachidonic acid has a profound effect on weight gain in ethanol-fed animals, lowering it in one study (Goheen et al, 1983), whilst increasing it in another (Goheen et al, 1980).
These inconsistencies, and the lack of data on blood ethanol levels or precise ethanol intakes (in g/kg/day) in these studies sheds some doubt on the findings. In addition, it should be asked whether arachidonic acid might protect against fatty liver by inhibiting ethanol-induced lipid peroxidation, as arachidonate is an effective antioxidant in its own right (Slater, 1981). There is one study to show that prostaglandin El protects against alcoholic fatty liver in rats, although no mechanism to explain this effect has been described (Wilson et al, 1973).

Thus, although there is some evidence that ethanol consumption changes the fatty acid composition of hepatic membrane phospholipids, the mechanisms underlying this effect are not clearly defined, and the possible importance of these changes in fatty liver production is not clear. More studies on the mechanisms by which arachidonic acid and prostaglandin El protect against fatty liver are needed. It is evident that prostaglandins may have a non-specific cytoprotective effect, as prostaglandin \( E_2 \) protects against carbon tetrachloride hepatotoxicity, as well as protecting the stomach against a variety of noxious agents (Stachurcha et al, 1981; Burke, 1981).

1.3.7. Carbohydrate Deprivation and Alcoholic Fatty Liver

Rao and Larkin (1984) have suggested that alcoholic fatty liver is the consequence of a synergistic effect between ethanol toxicity and the depleted carbohydrate content of the alcohol-containing liquid diets used to induce fatty liver in experimental animals. Some support for this hypothesis comes from studies in which
supplementation of the diets with dihydroxyacetone phosphate, riboflavin and pyruvate prevented fatty liver (Stanko et al, 1978; Goheen et al, 1981). It was originally thought that these natural metabolites might prevent fatty liver through acting as hydrogen acceptors, thereby correcting the ethanol-induced hepatic redox-state change. However, it was recently reported that only pyruvate was necessary out of the 'cocktail' described above to prevent fatty liver formation (Rao et al, 1984). Pyruvate or glycerol, at a concentration of 22 g/l added to the Lieber-DeCarli liquid diet, significantly decreased ethanol-induced steatosis in rats. This supplementation does, however, reduce the proportion of ethanol-derived calories in the diet from 36% to 33.5%, so this could account for the decreased fat accumulation in view of the earlier discussion on animal models of alcoholic liver injury. Isocaloric substitution of carbohydrate (normally 47% of the total calories in the control Lieber-DeCarli diet) with ethanol will leave ethanol-fed animals with only 11% of their calorie intake as carbohydrate, so Rao et al (1984) proposed that the fatty liver was a consequence of long-term carbohydrate deprivation, although no detailed mechanisms by which this might cause steatosis were described. Furthermore, the liquid diet employed in this current thesis still contained 29% of its total calorie content as carbohydrate after ethanol had replaced 36% of the calories, yet fatty liver was still produced after long-term ethanol feeding. Thus, there is some doubt surrounding the validity of this recently-proposed mechanism for the pathogenesis of alcoholic fatty liver.
1.4. MECHANISMS OF ALCOHOL-INDUCED FATTY LIVER: CONCLUDING REMARKS

It will be clear from the above discussion that there are nearly as many hypotheses concerning the pathogenesis of alcoholic steatosis, and alcoholic liver disease in general, as there are workers in this field. The redox-state theory (increased NADH:NAD ratio) is probably the most widely-quoted mechanism for the production of fatty liver, and is a very neat explanation for this lesion, but is not convincingly proven experimentally. It is still not clear whether alterations in lipid metabolism that have been attributed to the redox-state changes, chiefly impaired fatty acid oxidation, are important in the pathogenesis of fatty liver. In the case of decreased fatty acid oxidation after ethanol intake, it is not clear whether this arises through some other mechanism, such as direct acetaldehyde toxicity or lipid peroxidation. Ethanol-induced lipid peroxidation seems a feasible mechanism, in view of the protective effects of antioxidants that have been reported, and the fact that other hepatotoxins, such as halogenated hydrocarbons, are thought to act through a free radical-mediated mechanism. However, no precise mechanism by which ethanol ingestion leads to increased free radical generation in the liver has been described, as has been for carbon tetrachloride. The possible relationship between ethanol-induced centrilobular hypoxia and lipid peroxidation remains to be investigated. Another criticism of the lipid peroxidation theory might be that measurement of increased peroxidation after ethanol
administration may not be a primary effect of the toxin or its metabolism. Compromised tissues tend to show increased evidence of peroxidation secondary to other damaging events having occurred previously, and this may be the case for ethanol (Walliwell and Gutteridge, 1984). However, the effects of antioxidants on alcoholic fatty liver in rats suggest that it may be an important stage nevertheless. The possible central role of acetaldehyde in these mechanisms should not be understated, and this substance's direct toxic actions may certainly have a role to play. What is needed in future studies is an evaluation of the inter-relationship between some of these proposed mechanisms. For example, is mitochondrial injury due to continued exposure to ethanol-derived acetaldehyde, or due to peroxidative damage, or both? Similarly, is impaired hepatic protein secretion attributable to one or both of these factors?

Many of the other mechanisms described (eg: enzyme induction) may be responses to other effects of ethanol on metabolic or hormonal balance rather than being primary mechanisms by which liver damage or steatosis occurs. Taking all the evidence into account, it seems likely that ethanol-induced fatty liver, and possibly some of the latter stages of alcoholic liver disease seen in man, are due to toxic effects mediated by acetaldehyde, including lipid peroxidation, impaired protein secretion, and possibly altered mitochondrial function, being the most important. It seems that acetaldehyde may exert these effects both directly, through covalent interaction with specific cellular proteins, and also by enzymatic mechanisms that
could cause increased free radical production. As a result, the 'redox-state' explanation for fatty liver may not be the most important underlying mechanism.

1.5. STUDIES ON HEPATOPROTECTIVE AGENTS IN ALCOHOLIC FATTY LIVER

There have been numerous reports on the ability of a wide range of substances to inhibit the formation of fatty liver after acute or chronic ethanol administration. As a result of these studies, various hypotheses concerning the mechanisms of fatty liver production have been proposed. In this section, these protective agents will be briefly presented along with possible mechanisms by which they act. A few general points on the interpretation of studies with protective agents should be made here though. Many agents have only been described to protect against acute ethanol hepatotoxicity. As described earlier, ethanol metabolism is a requirement for fatty liver to occur after acute ethanol dosing. Not all the studies described have presented data on the effect of the protective substances on ethanol metabolism, so that interpretation of the results is difficult. In the case of pyrazole (see Section 1.2.2.), inhibition of ethanol metabolism can lead to conflicting results between acute and chronic experiments of fatty liver production, even though it seems similar mechanisms may account for both types of fatty liver. Effects of protective agents on factors that may affect the extent of fatty infiltration of the liver, independent of the proposed mechanism
of action of the compound, are not always assessed, eg: effects on peripheral fatty acid release. In chronic studies, effects of compounds on dietary (and hence ethanol) intake are not always reported, any large reduction in the daily ethanol intake (expressed in g/kg/day) obviously affecting the degree of fatty infiltration observed. Any effects of the compounds on ethanol metabolism or absorption should be determined from blood ethanol measurements, although these are not always carried out. The various substances that have been reported to prevent alcoholic fatty liver in rats are briefly described below.

1.5.1. Substances Other Than (+)-Catechin Compounds

(i) Lipotrophic cocktail: Addition of pyruvate, dihydroxyacetone phosphate and riboflavin to high-fat ethanol-containing liquid diets of rats was found to prevent the chronic fatty liver (Stanko et al, 1978). These authors proposed that these substrates acted as hydrogen acceptors, correcting the elevated NADH:NAD ratio in the liver. However, no redox-slate measurements were made in this study, and it has subsequently been discovered that only pyruvate is necessary for fatty liver prevention (Rao and Larkin, 1984). The possible implications of this latter finding have been discussed in detail above (Section 1.3.7.)

(ii) Barbiturates: Phenobarbital has a partial protective effect against the acute ethanol-induced fatty liver (Vincenzi et al, 1967), when given in a dosage regime known to induce cytochrome P450. However, no blood ethanol levels were determined in this study, and it
has subsequently been shown that sodium diethylbarbiturate prevents the acute ethanol-induced effects on lipid metabolism in mouse liver through inhibition of alcohol dehydrogenase (Rawat and Kuriyama, 1972).

(iii) **Nicotinic acid:** Early studies showed that pre-treatment of rats with nicotinamide corrected the disturbed NADH:NAD ratio after acute ethanol administration, but did not affect the triglyceride accumulation under the same conditions (Reboucas and Isselbacher, 1961). Nicotinic acid also corrects the redox-state after acute ethanol dosing, but does prevent the hepatic lipid accumulation occurring. This effect is probably due to the vitamin inhibiting alcohol dehydrogenase activity and peripheral lipolysis in ethanol-treated rats (Greenberger et al, 1965; Baker et al, 1973).

(iv) **Antihistamines and SKF-525A:** The antihistamine drugs chlorcyclizine and promethazine, as well as the 'hepatic microsomal enzyme stimulant' SKF-525A prevented the hepatic triglyceride accumulation after a large dose of ethanol (6 g/kg orally) (Woolles, 1968). SKF-525A can also act as a cytochrome P450 inhibitor, and no mechanism of action for the protective effect of this substance was proposed in this study. However, promethazine protects against carbon tetrachloride toxicity, partly due to its antioxidant action, although physiological effects of the drug (lowering of body temperature) also influence carbon tetrachloride metabolism (Reddrop et al, 1983). No determination of the effects of promethazine on blood ethanol levels was made in the Woolles (1968) study, although
more detailed study of the action of chlorcyclizine showed that this drug did not affect ethanol metabolism after the large ethanol dose used to produce fatty liver, and furthermore, chlorcyclizine prevented the ethanol-induced decrease in fatty acid oxidation \textit{in vivo} (Wooles and Weymouth, 1968). It was suggested that promethazine and chlorcyclizine might act through a free radical-scavenging action, although in view of the data on SKA-525A it was proposed by the authors that stimulation of microsomal enzyme activity was the more likely mechanism of protection for these drugs, since SKF-525A does not possess a benzyl carbon atom, thought to confer antioxidant activity (Wooles, 1968). However, more detailed studies on the free radical scavenging action of these compounds, their interaction with ethanol metabolism and hepatic microsomal enzyme activity are lacking.

(v) \textbf{Antioxidants:} A detailed discussion of the protective action of a number of antioxidants against ethanol-induced fatty liver, and the possible role of lipid peroxidation in this process, is given in Section 1.2.6. However, more detailed studies on the \textit{in vitro} antioxidant activity of some of these compounds, their ability to inhibit ethanol-induced lipid peroxidation \textit{in vivo}, and their interaction with ethanol metabolism are required, in order to establish that these compounds do exert their protective action through inhibition of lipid peroxidation.

(vi) \textbf{Clofibrate:} The hypolipademic drug ethyl $\alpha$-p-chlороphenoxyisobutyrate (Clofibrate) has been shown to inhibit both the acute (Brown, 1966) and chronic (Spritz and Lieber, 1966) forms of
alcoholic fatty liver. The mechanisms of protection may be different for the two experimental situations. After acute ethanol dosing, the drug prevents the increased \(\alpha\)-glycerophosphate:dihydroxyacetone phosphate ratio, and may also inhibit peripheral lipolysis (Kahonen et al., 1971; Kahonen et al., 1972; Rifkind, 1966). The former action could be achieved simply by inhibition of ethanol metabolism, yet no data on the effect of the drug on ethanol elimination was given in these studies.

In the chronic study (Spritz and Lieber, 1966), the drug may act through one or both of its two more recently described properties. Firstly, clofibrate is a potent inducer of peroxisome proliferation and greatly enhances peroxisomal \(\beta\)-oxidation of fatty acids, so that this may overcome any ethanol-induced block in mitochondrial fatty acid oxidation, thus preventing esterification of excess fatty acyl moieties into triglycerides (Eliassen and Osmundsen, 1984). Secondly, clofibrate feeding enhances hepatic aldehyde dehydrogenase activity in rats, thus possibly protecting against toxic effects of acetaldehyde (Kramar et al., 1984).

(vii) Propylthiouracil: The concept of the ethanol-induced hypermetabolic state, its possible relationship to centrilobular hypoxia, and the possible role of antithyroid drugs in counteracting these effects has already been described in detail (Section 1.3.5). This theory was supported by the protective effect exerted by propylthiouracil against experimental alcoholic hepatitis in rats (Israel et al., 1975), and one clinical trial showed some benefit of
the drug in patients with alcoholic hepatitis (Orrego et al, 1979). However, hypothyroidism does not protect against the development of alcoholic liver injury (Soff and Resnick, 1981), and propylthiouracil also protects against the hepatotoxicity of acetoaminophen and carbon tetrachloride, independently of any antithyroid action, as discussed previously (Linscheer et al, 1980; Orrego et al, 1976). These propylthiouracil studies have not been followed up as yet with studies on the action of other antithyroid drugs in the same experimental models.

(viii) Penicillamine and other thiol compounds: There has been much interest in the use of penicillamine in the treatment of hepatic cirrhosis due to its ability to inhibit lysyl oxidase, so limiting the ongoing fibrotic process (Orbison, 1975). Its properties as a copper-chelating agent have led to its use for the treatment of Wilson's disease (Walshe, 1960). There is also evidence to suggest that this compound may be effective in preventing fatty liver, due to its ability to interact with acetaldehyde, and prevent acetaldehyde-induced mitochondrial injury in vitro (Cederbaum and Rubin, 1976b), in a manner similar to that reported for cysteine (Cederbaum and Rubin, 1976a). These thiol compounds appear to interact with acetaldehyde through their free sulphydryl and amino groups to form hemi-acetals, cysteine derivatives in which the amino or sulphydryl groups are blocked (eg: 5-methylcysteine or N-acetylcysteine) not being effective in these in vitro studies. The potent ability of thiol compounds to prevent these acetaldehyde
induced mitochondrial impairments has only been partially followed up by in vivo studies on their influence on ethanol-induced liver injury. Penicillamine has been shown to lower blood acetaldehyde levels after ethanol dosing in vivo (Nagasawa et al., 1977), although the acetaldehyde concentrations reported were very high and may reflect artefactual formation problems in the assay procedure (Eriksson, 1980). Macdonald et al. (1977) showed that cysteine, methionine and β-mercaptoethylamine improved survival time in mice given lethal doses of ethanol, although no objective measures of liver injury were carried out. Cysteine protects against LD_{90} doses of acetaldehyde given to rats (Sprince et al., 1975), but again this was not related to measures of hepatotoxicity. 2-mercaptopropionylglycine and reduced glutathione afforded partial protection against the increase in hepatic triglyceride resulting from repeated daily ethanol dosing (3 g/kg orally) (Torrelli et al., 1978). Cysteine and 2-mercaptopropionylglycine protect against the acute ethanol-induced fatty liver in rats, this effect being associated with lowering of hepatic acetaldehyde concentrations (Hirayama et al., 1983).

There have been no studies reported on the ability of the thiol compounds mentioned above to inhibit the fatty liver induced by feeding of ethanol in a liquid diet. Lieber and DeCarli (1966), however, found that addition of methionine to the liquid diet regime prevented the chronic alcoholic fatty liver in rats. The mechanism of protection of methionine is not clear, but it may involve conversion to cysteine, as methionine itself cannot interact with
acetaldehyde, as it does not possess a free sulphhydryl group. Methionine has also been shown to protect against ethanol-induced lipid peroxidation (Shaw et al, 1981), but again, this may be through a direct interaction with free radicals, or through conversion to cysteine and lowering of liver acetaldehyde levels as a result. Many of these thiol compounds (cysteine, penicillamine, 2-mercaptopropionylglycine, methionine) have free radical scavenging (antioxidant) properties in their own right, so this may account, in part, for some of the reported protective effects (Slater, 1981).

Cysteine and methionine may influence hepatotoxicity through their role as glutathione precursors. Thus, more detailed and comprehensive studies on the effect of thiol compounds on ethanol hepatotoxicity are required before their mechanism of action is completely understood.

(ix) Arachidonic acid and prostaglandins: Dietary arachidonic acid protects against chronic alcohol-induced fatty liver in mice and rats (Goheen et al, 1980; Goheen et al, 1981; Goheen et al, 1983; Karpe et al, 1984). Prostaglandin E1 will also prevent the chronic alcoholic fatty liver (Wilson et al, 1973). The possible relationship between these observations and altered essential fatty acid and prostaglandin metabolism during ethanol consumption has already been discussed (Section 1.3.6). As with many of the other hepatoprotective agents described, the effect of these agents on ethanol intake, absorption and elimination has not always been completely investigated. In the case of arachidonic acid, the daily
dose consumed in some experiments is quite considerable (up to 1 g/kg/day), and it has to be asked whether at this dose, a possible antioxidant effect of the acid is responsible for its protective action.

(x) \(\alpha\)-Adrenergic agents: The protective actions of \(\alpha\)-adrenergic blockers (dibenamine and ergotamine) against acute alcoholic fatty liver (Brodie et al., 1961) have already been mentioned (Section 1.3.2), and the possible relevance of these effects to the role of peripheral lipolysis in fatty liver production discussed in detail.

(xi) Lipotropic factors: Methionine and choline protect against the chronic ethanol-induced fatty liver in rats (Lieber and DeCarli, 1966). This may be considered a pharmacological action of these substances, since the levels added to the alcohol-containing liquid diets was greatly in excess of that required to prevent hepatic steatosis arising from choline or methionine-deficient diets alone. Carnitine supplementation of alcoholic liquid diets also reduces the triglyceride accumulation after long-term ethanol intake in rats (Sachan and Rhew, 1983). The daily carnitine intake of animals in the latter study was of the order of 1.5 g/kg/day, so once again, a pharmacological action of the substance is probably responsible for its anti-steatotic effect. Studies on the mechanism of action of these substances are lacking, although a possible mechanism for methionine has already been discussed above. Both carnitine and choline have the ability to react with acyl groups, so may possibly act as acetaldehyde traps, but there is no experimental evidence to
support this. It is unlikely that carnitine prevents fatty liver by overcoming an ethanol-induced block in mitochondrial fatty acid uptake as a result of carnitine deficiency, as the livers of alcohol-fed rats in fact show an increased carnitine content (Sachan and Rhew, 1983).

1.5.2. (+)-Catechin (cyanidanol-3) and 3-Palmitoyl-(+)-Catechin

The subject of this thesis is the investigation of the effect of (+)-catechin (3',4',5,7-tetrahydroxyflavan-3-ol), and its more lipid soluble ester, 3-palmitoyl-(+)-catechin, on ethanol hepatotoxicity in the rat. Many of the studies on the mechanism of action of the drugs have employed the latter substance, due to its greater potency at preventing fatty liver.

(i) Chemical and physical properties: The chemical structures of (+)-catechin and 3-palmitoyl-(+)-catechin are shown in Fig. 1.3. (+)-Catechin is a naturally-occurring benzopyran bioflavanoid substance. 3-Palmitoyl-(+)-catechin is a chemically synthesised derivative of (+)-catechin. Both compounds are poorly soluble in water, but show good solubility in alcohols (eg: 50% solubility in methanol) and most organic solvents. Both substances are stable at room temperature for several years and are not affected by exposure to light.

(ii) Absorption, distribution, metabolism and excretion studies: The findings of various studies which have investigated the metabolism of (+)-catechin and 3-palmitoyl-(+)-catechin can be summarised as follows. Both substances are well absorbed after oral dosing, up to 80% of the administered dose being taken up. Absorption is rapid,
Fig. 1.3. **CHEMICAL STRUCTURES OF 3-PALMITOYL-(+)-CATECHIN AND (+)-CATECHIN**
particularly in the case of 3-palmitoyl-(+) catechin. Both compounds undergo extensive first pass metabolism in the liver, and there is a rapid disposition of both substances in the whole organism. About 75% of the 3-palmitoyl-(+) catechin in plasma is protein-bound, as opposed to 50% in the case of (+) catechin itself. After oral administration of radiolabelled [U-\(^{14}\text{C}\)]-3-palmitoyl-(+) catechin and [U-\(^{14}\text{C}\)]-(+) catechin, the highest concentrations of radioactivity are found in the liver (about four times the concentration found in the blood). In the case of [U-\(^{14}\text{C}\)]-(+) catechin there is a rapid increase in liver radioactivity with a peak at one hour after dosing, followed by a rapid decrease. This peak level of radioactivity after 3-palmitoyl-(+) catechin dosing in rat liver is not achieved until 4-12 hours after oral dosing, and there is considerable persistence of radioactivity in liver tissue, possibly as a result of protein binding of the drug. Significant levels of radioactivity are recovered in the kidney after administration of both compounds. Levels of radioactivity in the lungs, heart, muscles and testes are lower than blood radioactivity levels for both substances, and no radioactivity is detectable in the brain of rats after administration of either drug.

In the rat, the metabolism of (+) catechin seems to involve a methylation step at the 3' position on the catechol ring, conjugation of this metabolite to glucuronic acid and sulphate taking place before excretion in the bile. 5-Adenosylmethionine is thought to be the source of the donated methyl group, the transfer being catalysed by
catechol-O-methyl transferase. Reabsorption and enterohepatic circulation of these metabolites is thought to take place, most of the drug being excreted in the urine as 3'-0-methyl-(-)-catechin glucuronide or 3'-0-methyl-(+)-catechin sulphate (Shaw and Griffiths, 1980; Hackett et al, 1982). Earlier studies have shown that the intestinal microflora can act on the biliary metabolites of (+)-catechin to produce the ring scission products m-hydroxyphenylpropionic acid, p-hydroxyphenylpropionic acid, 8-(3-hydroxyphenyl)-γ-valerolactone and 8-(3,4-dihydroxyphenyl)-valerolactone. These metabolites are reabsorbed and excreted in the urine (Das and Sothy, 1971). There is some species variation in the reabsorption and elimination route of (+)-catechin metabolites. In the rat, 66% of an oral dose of [U-14C]-(+)-catechin is eliminated in the urine, 24% being recovered in the faeces, whereas in the marmoset, 22% of the dose is excreted in the urine, and 70% in the faeces (Hackett et al, 1982).

The metabolism of 3-palmitoyl-(+)-catechin is very similar to that of (+)-catechin, once the drug has been hydrolysed to form the parent compound. This initial de-esterification appears to be the rate-limiting step in the overall metabolism of the compound and could account for the delayed elimination of the drug when compared with (+)-catechin (Hackett and Griffiths, 1982). (+)-Catechin is rapidly eliminated, serum levels falling rapidly after oral dosing. After dosing with [U-14C]-(+)-catechin, 90% of the radioactivity is excreted in the first 24 hours after dosing. After oral administration of
3-palmitoyl-(+)-[U-^{14}C]-catechin, steady levels of radioactivity are achieved in the serum in the first 24 hours after dosing, and it takes 17 days for greater than 90% of the administered dose to be recovered in the urine, faeces and expired carbon dioxide. $^{14}$C persists in certain tissues, and 28 days after dosing, 3-7% of the dose was still present in the animal body (Hackett and Griffiths, 1982). Unlike (+)-catechin, some retention of radioactivity after 3-palmitoyl-(+)-[U-^{14}C]-catechin dosing occurs in the adipose tissue. Studies in human volunteers have confirmed the animal findings that glucuronides of (+)-catechin and 3'-O-methyl-(+)-catechin are the major urinary metabolites of (+)-catechin. After [U-^{14}C]-(+)-catechin dosing in man, 55% of the dose was recovered in the urine in the first 24 hours. Ring scission product metabolites accounted for less than 10% of the urinary metabolites of (+)-catechin in this study (Hackett et al, 1983). No human data on the metabolism and excretion of 3-palmitoyl-(+)-catechin has been reported.

(iii) Toxicity studies: Both compounds are non-toxic in animals at the normal doses employed in pharmacodynamic studies. Acute studies in rats show that greater than 5 g/kg orally of the drugs have to be given before any deaths occur. (+)-Catechin is also safe given by the intraperitoneal route, but 3-palmitoyl-(+)-catechin has an LD$_{50}$ of about 80 mg/kg in rats when given by this route. Chronic toxicity tests in rats with 3-palmitoyl-(+)-catechin showed that daily oral dosing with up to 900 mg/kg of the drug for two weeks was without effect, but repeated dosing with 2700 mg/kg led to deaths within one
week. Studies in dogs showed that daily oral doses of 3-palmitoyl-(-)-catechin (400 mg/kg) reduced food intake and caused weight loss.

(iv) Animal pharmacodynamic studies: The first study outlining the potential hepatoprotective properties of (±)-catechin was that of Gajdos et al (1972). This showed that the drug prevented hepatic ATP depletion induced by ethionine or malonic acid administration. The drug also protected against hepatic steatosis induced by a low-protein diet, orotic acid or ethanol. In the case of the latter two toxins, correction of the reduced NAD\textsuperscript{+}:NADH ratio and lowered ATP levels by (±)-catechin was reported. The ethanol experiment involved twice daily intraperitoneal injection of a 20% (w/v) solution for one week, each dose being 1.4 g/kg body weight. This regime apparently caused hepatic steatosis, although no saline-dosed controls were employed. Daily subcutaneous injection of (±)-catechin (50 mg/kg) or daily oral dosing of the drug (200 mg/kg) caused approximately 25-30% lowering of the total hepatic lipid concentration in ethanol-treated animals. No controls were employed. Increases in the NAD\textsuperscript{+}:NADH ratio and hepatic ATP level due to (±)-catechin were reported, but these were measured in tissue samples which had simply been removed into ice-cold buffer, no freeze-clamping being performed as would normally be expected for measurement of these labile metabolites. Thus, this early study suggested a possible hepatoprotective action for (±)-catechin against ethanol hepatotoxicity, although the model employed would not be considered a valid one for chronic ethanol-induced fatty liver, and
other methodological shortcomings indicated further work was required. Correction of the altered hepatic redox-state by (+)-catechin appeared to be the mechanism of action in this study.

Danni et al (1977) reported in vitro studies indicating that (+)-catechin was a potent free radical scavenger (antioxidant). In vitro, low concentrations of the drug (10-100 μM) protected microsomes against stimulation of lipid peroxidation by carbon tetrachloride, although high doses (1.5 g/kg i.p.) were required to protect against carbon tetrachloride hepatotoxicity in vivo (Slater and Scott, 1981). The reactive radical formed from carbon tetrachloride is thought to be \( \text{CCl}_3\text{O}^- \), (+)-catechin having an \( EC_{50} \) (50% inhibitory concentration) against in vitro peroxidation induced by this radical of 26.9 μM. (+)-Catechin reacts even more readily with hydroxyl radicals generated by NADPH-ADP/Fe\(^{2+}\), its \( EC_{50} \) for in vitro peroxidation induced in microsomes by this system being 9.6 μM (Slater, 1968; Slater and Scott, 1981). (+)-Catechin also shows reactivity with superoxide anions generated by NADH and phenazine methosulphate (Ponti et al, 1978), the \( EC_{50} \) for nitroblue tetrazolium reduction by this system being 215 μM. All the quoted \( EC_{50} \) values are lower still if 3-methyl- (+)-catechin or 3-butyl- (+)-catechin are substituted for (+)-catechin itself, indicating that lipid solubility is important in protecting liver membranes against peroxidation. These observations, in combination with the pharmacokinetic considerations already discussed, were the underlying reason for development of 3-palmitoyl- (+)-catechin as a more potent derivative of (+)-catechin.
for in vivo studies.

There have been several studies on the protective action of (+)-catechin against hepatotoxins other than ethanol. Some studies on carbon tetrachloride in isolated rat liver microsomes have already been described, and the drug protects isolated hepatocytes against the 'blebbing' (observed by electron microscopy) and release of intracellular proteins and enzymes into the incubation medium induced by either carbon tetrachloride or phalloidin (Perrissoud et al, 1981). In vivo protection against the hepatotoxicity of bromobenzene, thioacetamide, vinylidene chloride and halothane in combination with hypoxia has been reported (Siegers, 1981; Siegers et al, 1983). (+)-Catechin protects against galactosamine-induced inhibition of hepatic protein synthesis and secretion in rats (Reutter and Hassels, 1980). No precise mechanism of action of the drug against these hepatotoxins was proposed in these studies, most ascribing protection to a relatively non-specific membrane stabilisation and/or free radical scavenging activities. Videla et al (1981), suggested that the latter mechanism might account for the protective action of (+)-catechin against ethanol hepatotoxicity. They showed that the drug (400 mg/kg i.p.) given at the same time as ethanol (5 g/kg p.o.) prevented the decrease in hepatic glutathione levels and increased lipid peroxidation of liver lipids (measured as diene conjugates) after ethanol administration. These findings were not correlated with any hepatic triglyceride measurements, however, and several criticisms can be levelled at these authors method of measuring diene
conjugates. These were determined in whole liver homogenates rather than subcellular fractions, and the absorbance of the lipid extract was determined at 233nm in relation to the protein content of the homogenate, no lipid determination being carried out. If it is assumed that ethanol dosing caused an increase in liver triglyceride content, the increase reported in diene conjugates per milligram liver protein may simply reflect increased end absorption of hepatic lipids due to triglyceride accumulation, rather than true diene absorption. Thus, it cannot be firmly established from this study whether ethanol actually induced lipid peroxidation.

There are relatively few studies on the effects of (+)-catechin on drug metabolism. A single large dose of the drug (200 mg/kg i.p.) given 13 hours prior to sacrifice, decreased the cytochrome P450 concentration by 24%, aminopyrene and p-nitroanisole metabolism being reduced by similar degrees, but marked inhibition (> 50%) of ethoxycoumarin and ethoxyresorufin metabolism was found. This suggests a preferential inhibition of metabolism mediated by cytochrome P448. In vitro, (+)-catechin is a powerful inhibitor of epoxidation of carbamazepine in rat liver microsomes (Steffen et al, 1981). Acute and chronic treatment with (+)-catechin has been found to inhibit glutathione-S-transferase activity towards epoxide or acyl substrates (Younes et al, 1982). (+)-Catechin does not cause induction of any drug-metabolising enzyme activities in the liver or increase hepatic cytochrome P450 concentrations after long-term treatment.
Some immune-stimulating properties of (+)-catechin have been reported that may account for its ability to induce seroconversion (i.e., elimination of hepatitis-B surface antigen) in patients with various forms of chronic hepatitis. In leucocyte migration inhibition tests, the drug amplifies the cell-mediated immune response with the antigen tuberculin and HBsAg (Vallotton and Frei, 1981).

(+)-Catechin has been shown to interact with collagen metabolism. In vitro, collagen that has been treated with the drug is resistant to the action of collagenase, and degradation of soluble collagen is thus decreased. Detailed studies have shown that 6-7 molecules of (+)-catechin are bound per 1-chain of collagen in these studies, and that the drug promotes cross-linking of collagen (Rao et al, 1981; Kuttan et al, 1981). In view of these properties, it has been proposed that the drug be used for the treatment of diseases where the formation of a normal collagen matrix is impaired, such as osteogenesis imperfecta (Pontz et al, 1982).

An unrelated property of (+)-catechin that has been reported is its ability to inhibit gastric ulcer formation in rats as a result of its inhibition of histidine decarboxylase. This may make the drug an alternative to the histamine-H2 receptor antagonist drugs that are conventionally used to treat gastric and duodenal ulceration in man (Parmar and Ghosh, 1981).

Some basic in vitro studies on metabolic effects of (+)-catechin have yielded some interesting findings that may be relevant to the
correction of ethanol-induced redox-state changes reported by Gajdos et al, (1972). In isolated rat hepatocytes, (+)-catechin inhibits glycogenolysis and stimulates net glycogen production. The drug was found to enhance the activities of glycogen synthase and inhibit glycogen phosphorylase activities, in accord with the observed effects on total glycogen production (Nyfeler et al, 1983). These findings do not correlate with the mechanism proposed by Gajdos et al (1972) to explain the correction of the increased NADH:NAD ratio and lower ATP levels in the livers of ethanol-dosed rats. These authors proposed that the drug stimulated glycolysis, thereby generating more pyruvate to reoxidase NADH by way of the lactate dehydrogenase reaction, ATP being synthesised in the process as a result of substrate level phosphorylation in the glycolytic pathway.

(v) Clinical studies on (+)-catechin: Several clinical trials of (+)-catechin in the treatment of hepatitis have been reported. In patients with acute viral hepatitis the drug accelerated the disappearance of hepatitis B surface antigen from the blood, lowered serum bilirubin and relieved various clinical symptoms of the illness (Blum et al, 1977). Piazza et al (1983) found greater reductions in plasma transaminase activities and serum bilirubin levels in patients with chronic persistent hepatitis when drug-treated subjects were compared with placebo-dosed controls. However, one of the largest studies of the drug in hepatitis patients found no improvement in any of these parameters in drug-treated patients (Schomerus et al, 1981).
Studies on the influence of the drug on alcoholic liver disease have also failed to give clear-cut findings. Again, improvements in serum liver function tests have been reported, although improvement is often observed in the placebo group as well in these studies, as a result of patients abstaining or reducing their alcohol intake during the study (Conn, 1983). One Italian study in alcoholic cirrhotics showed consistent improvements in liver function tests and clinical symptoms such as anorexia and abdominal discomfort (Palmas, 1981). In another study carried out in pre-cirrhotic alcoholics, no significant effect of the drug could be found (Henning, 1981). Another study suggested that the drug was of benefit in the early stages of alcoholic liver disease, but not once established lesions (cirrhosis) had been induced (Ugarte et al, 1981).

Two trials of the drug in alcoholic liver disease have been completed in the United Kingdom. The first, undertaken at the Royal Free Hospital, was a three-month, randomised, double-blind, placebo-controlled study in 40 patients with pre-cirrhotic liver disease. Patients were classified as either 'abstinent' or 'drinking' during the study, however in neither group did the drug have any significant effect on histological appearance of the liver, serum liver function tests or the mean corpuscular volume (Colman et al 1980). The study of World et al (1984) was of six months duration. Interestingly, the drug-treated group in this study had worse liver function tests at the outset, drank consistently more during the trial, yet after the fourth
week of treatment, showed consistently lower gamma-glutamyl transpeptidase and aspartate aminotransferase activities, although these changes were not statistically significant. This suggests that the drug may have had a slight protective effect against ongoing liver damage in drinking alcoholics. Thus, there is some confusion as to the clinical effectiveness of the drug at the present time. It should be noted that the doses of the drug employed in these studies (1.5-2.0 g/day or about 25 mg/kg/day) are rather less than those that are effective at influencing liver function in animal models. Therefore, more promising results might be achieved if 3-palmitoyl- (+)-catechin had been used in place of (+)-catechin in these studies, in view of the more favourable pharmacokinetic and pharmacodynamic properties of this derivative discussed earlier. No clinical studies on 3-palmitoyl- (+)-catechin have been reported to date.

1.6. PROPOSED STUDIES

It will be clear from the discussion above that (+)-catechin has a number of properties that may be of benefit in the treatment of alcoholic liver disease. Modulation of the redox-state, free radical scavenging and immunological effects are probably the most important of these. In terms of preventing experimental alcoholic liver injury in rats (ie: fatty liver), the study of Gajdos et al (1972) suggests
redox-state correction may be a possible mechanism, whereas Videla et al (1981) indicate that inhibition of ethanol-induced lipid peroxidation could be the mode of action. These mechanisms are somewhat presumptuous since neither study employed a satisfactory model of chronic ethanol-induced steatosis to determine whether the drug was effective. Thus, the aim of the experimental work was, firstly, to establish the protective properties of (+)-catechin and 3-palmitoyl-(+)-catechin in valid models of acute and chronic (using a liquid diet method of ethanol administration) fatty liver in rats. Having established the effect of the drugs in these experimental models, the mechanism by which they may afford any protection will be investigated by comparing the actions of (+)-catechin or 3-palmitoyl-(+)-catechin with those of certain reference compounds with specific properties (eg: antioxidants or compounds which modulate the hepatic redox-state) in the same experimental situations. This approach should enable some distinction to be drawn between the two mechanisms of action proposed for the drug by Gajdos et al (1972) and Videla et al (1981) respectively.

The experiments presented here have concentrated on the ability of the (+)-catechin compounds to prevent steatosis by prophylactic means. In both the chronic and acute experiments, a number of parameters have been included in the protocols in order to gain as much information as possible about, for example, the relationship of redox-state changes to alterations in hepatic lipid disposal, and the
effects of reference compounds in this respect. This approach should, hopefully, lead to a clear picture of the mode of action of the (+)-catechin compounds emerging.
Chapter 2

MATERIALS AND METHODS

2.1 CHEMICALS, DIETS AND REAGENTS

(i) Liquid Diet Constituents: 'Complan' (plain flavour) was supplied by Glaxo-Farley Foods, Plymouth, Devon, England. 'Orovite-7' was supplied by Bencard, Greenford, Middlesex. Casein (white, soluble, cat. no. 44016 6T) was supplied by B.D.H. Chemicals, Poole, Dorset. Glucose (anhydrous) was supplied by Sigma Chemical Co., Poole, Dorset. The ethanol used in all experiments was 100% absolute alcohol supplied by James Burroughs Ltd., London.

(ii) Liver lipid assays: Total lipids were measured using test kits supplied by B.C.L. Ltd., Lewes, Sussex (cat. no. 124303). Triglycerides were measured using a colorimetric kit supplied by Sigma.

(iii) Tissue Metabolite Assays: Perchloric acid (60%, analar grade) was from B.D.H. Chemicals. All cofactors (NAD and NADH) were obtained in preweighed vials (cat. nos. 260-120 and 340-102 respectively) from Sigma. Lactate dehydrogenase (Type II, cat. no. L2500) and \( \beta \)-glycerophosphate dehydrogenase (Type I, cat. no. G6751) were also supplied by Sigma. 3-Hydroxybutyrate dehydrogenase (Grade II, cat. no. 127191) was supplied by B.C.L. Ltd. Florisil (100-200 U.S. mesh, chromatographic grade, cat. no. 15026) was from B.D.H. Chemicals.
(iv) Lipid Utilisation Studies: [U-$^{14}$C] palmitic acid (specific activity 403 mCi/mmol cat. no. CFB37), [1-$^{14}$C] palmitic acid (specific activity 49 mCi/mmol, cat. no. CFA23), and [$^{14}$C]-hexadecane internal standard (1 x $10^6$ dpm/g; cat. no. CFR6) were supplied by Amersham International Ltd., Amersham, Bucks. Hyamine 10X was obtained as a 10% (w/v) solution in methanol (scintillation grade) from B.D.H. Chemicals (cat. no. 14608 4B). Soluene-350 tissue solubiliser and Dimilume-30 liquid scintillation cocktail were supplied by Packard Instrument Co. Ltd., Caversham, Bucks. All organic solvents employed were of analar grade from B.D.H. Chemicals. Bovine serum albumin (fatty acid-free, cat. no. A6003) was from Sigma. Silica Gel 60 thin-layer chromatography plates (Merck No. 5715, 20 x 20 cm, layer thickness 0.25 mm) were supplied by B.D.H Chemicals. Barium hydroxide was obtained as a 0.3N solution (cat. no. 14-3) from Sigma, and was stored over Carbosorb (6-12 mesh, B.D.H. Chemicals) in a dessicator. Hydrogen peroxide (30% v/v) was also from B.D.H. Chemicals.

(v) Gas chromatography: Poropak-Q (50-80 mesh) and the Pressure-Lok A2 gas syringe were supplied by Chromatography Services Ltd., Hoylake, Wirral, Merseyside. Acetaldehyde and n-propanol were of 'Aristar' grade and supplied by B.D.H. Chemicals. In some experiments, blood or serum ethanol was determined enzymatically using kits supplied by B.C.L. Ltd., (cat. no. 123960)

(vi) Assays for in vitro Antioxidant Activity: Phenazine methosulphate and nitroblue tetrazolium were from Sigma and were
stored dessicated in the dark at 0-5°C. NADPH was obtained from Sigma in preweighed vials (cat. no. 201-201). 2-Thiobarbituric acid (cat. no. T5500), ADP (Grade III, cat. no. A2754) and superoxide dismutase (cat. no. 57008, bovine liver, 3000 U/mg prot.) were from Sigma.

(vii) Serum Assays: Serum total protein and albumin were assayed using kits supplied by B.C.L. Ltd., (cat. no. 124281 and 263869 respectively). Rat albumin (cat. no. A6272) was from Sigma. Serum glutamate dehydrogenase activity was assayed using kits (cat. no. 124320) supplied by B.C.L. Ltd.

(viii) Drugs: Authentic samples of (+)-catechin and 3-palmitoyl-(+)-catechin were donated by the Research Dept., Zyma, Nyon, Switzerland. Preservative-free Naloxone hydrochloride powder was donated by Du Pont Ltd., Glenolden, Pennsylvania, U.S.A. Butylated hydroxytoluene (cat. no. B1378), methylene blue (cat. no. MB-1) and sorbitol (cat. no. S1876) were all from Sigma. N,N'-Diphenyl-p-phenylenediamine (DPPD) (cat. no. 66167) was supplied by B.D.H Chemicals.

(ix) Animals: All rats used in the present studies were supplied by the Animal Unit, University of Surrey, Guildford, Surrey. They were Male Wistar Albino Rats (University of Surrey strain), and body weight details are given later in each particular experiment. When animals were not maintained on the liquid diet regime, a standard chow diet (41B cube diet, Heygates Ltd.) and water was given.
2.2 INDUCTION OF ACUTE AND CHRONIC ETHANOL-INDUCED FATTY LIVER IN THE RAT

2.2.1 Acute studies:

The effects of protective agents on hepatic triglyceride accumulation was assessed after two different acute doses of ethanol. Firstly, the widely employed 6 g/kg oral ethanol dose was used. Ethanol was given by gastric tube as a 50% (v/v) solution in water, appropriate controls being dosed with isocaloric amounts of glucose (10.7 g/kg as an 71% (w/v) solution in water). Fasted rats were used. This ethanol dose has been used in a large number of studies on the acute effects of alcohol, and has the advantage that high blood ethanol levels are achieved (often over 400 mg/100 ml) and sustained for several hours after dosing. This leads to a large increase in the hepatic triglyceride level, the peak being observed between 10-18 hours after dosing (Mallow and Bloch, 1956; DiLuzio, 1958; Wooles, 1966). In the studies presented, animals were dosed at about 1800 h and sacrificed for liver triglyceride analysis 16 hours later, at 1000h the following day. This regime produced a histologically-observable centrilobular fat accumulation, similar to that seen after chronic ethanol feeding, although no evidence of hepatocellular necrosis was seen. Some of the disadvantages of this ethanol dose are that, due to its size, it does induce a prolonged narcosis and other profound physiological effects in the animals (eg: lowering of body temperature). The high concentration of the alcohol
<table>
<thead>
<tr>
<th>Ethanol dose</th>
<th>Parameter</th>
<th>Time after dosing (h)</th>
<th>Saline Control</th>
<th>Glucose Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0 g/kg p.o.</td>
<td>Liver Triglyceride (mg/g)</td>
<td>16</td>
<td>6.0 ± 1.2 (6)</td>
<td>7.6 ± 2.7+ (5)</td>
<td>18.6 ± 2.8* (10)</td>
</tr>
<tr>
<td></td>
<td>Blood Ethanol (mg/100 ml)</td>
<td>16</td>
<td></td>
<td></td>
<td>226 ± 88 (10)</td>
</tr>
<tr>
<td>2.0 g/kg i.p.</td>
<td>Liver Triglyceride (mg/g)</td>
<td>1.5</td>
<td>6.5 ± 1.6 (8)</td>
<td>8.8 ± 1.6+ (6)</td>
<td>14.5 ± 4.6* (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td>4.9 ± 0.9 (8)</td>
<td>N.D.</td>
<td>11.9 ± 4.5** (10)</td>
</tr>
<tr>
<td></td>
<td>Blood Ethanol (mg/100 ml)</td>
<td>1.5</td>
<td></td>
<td></td>
<td>219.8 ± 5.0 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.0</td>
<td></td>
<td></td>
<td>139.9 ± 11.3 (6)</td>
</tr>
</tbody>
</table>

All experiments were carried out in fasted male Wistar rats. Details of experimental procedures are given in the text. The number of determinations is given in parentheses. N.D. = not determined. + = n.s. vs saline; * = p < 0.001 vs saline; ** = p < 0.01 vs saline.
solution used causes gastric irritation and pyloric stenosis so that absorption of the ethanol may be highly variable and prolonged. These very high blood ethanol concentrations are often greater than those found in animals and humans during long-term ethanol intake, so the mechanisms of liver injury may not be identical between the two situations.

As discussed previously (Section 1.2.1) the mechanisms of liver injury after these large acute doses may differ from those operating during chronic ethanol intake (Badawy, 1985). In order to overcome some of these objections, a more moderate dose of ethanol given intraperitoneally was used. Two g/kg body weight ethanol was injected as a 20% (w/v) solution in saline, appropriate controls being dosed with equivalent volumes of saline or isocaloric solutions of glucose (i.e.: 3.5 g/kg as a 35% (w/v) solution in saline). This ethanol dose and route of administration gives very reproducible blood ethanol levels, which are similar to those found in animals fed the alcohol-containing liquid diet. Ethanol is metabolised quite rapidly, the blood levels returning to zero within 6-8 h after dosing, and the degree of narcosis and physiological impairment is much less than with the 6 g/kg oral dose. In fasted rats, this ethanol dose does however, cause a reproducible and significant increase in the hepatic triglyceride level during the intoxication period (Abrams and Cooper, 1976a; Abrams and Cooper, 1976b). Some typical liver triglyceride concentrations and blood ethanol concentrations after these acute ethanol doses are shown in Table 2.1. The values agree
well with those in the literature for identical experimental situations (Brodie et al., 1961; Woolfes, 1966; Abrams and Cooper, 1976a).

2.2.2. Chronic Ethanol Intake Studies:

A number of methods have been employed in an attempt to produce chronic alcoholic liver injury in rats, including administration of alcohol in the drinking water (Fry et al., 1978), or repeated daily dosing with ethanol (Torrielli et al., 1977). The drinking water method does not produce significant fat accumulation, and with both methods, producing identical nutritional intake between alcohol-treated and control animals is difficult. Thus, a model based on the nutritionally-adequate liquid diet, described by DeCarli and Lieber (1967), was developed to produce chronic fatty liver, and a pair-feeding technique has been used to ensure identical calorific and nutrient intake between alcohol-fed and control animals. The liquid diet devised was based on the liquid food supplement, 'Complan', in combination with casein, glucose and a commercial vitamin supplement ('Orovite-7'). This formula was chosen since the Lieber-DeCarli diet, in its commercial form, is very expensive and difficult to obtain in this country, and it is quite time-consuming to prepare from its basic ingredients. The 'Complan' diet is cheap by comparison, and is easy to prepare.
(i) **Dietary Regime**: A typical chronic experiment lasted for 36 days. The dietary regime was as follows:

Days 1-7: All animals receive control liquid diet, containing no ethanol, ad libitum.

Days 8-15: Ethanol-fed animals receive liquid diet in which 20% of the total calories are derived from ethanol, the ethanol calories replacing an isocaloric amount of glucose. Control animals are pair-fed control diet in which the ethanol calories are replaced by glucose.

Days 16-36: Ethanol-fed animals receive liquid diet in which 36% of the total calories are derived from ethanol, the control animals being pair-fed as described above.

(ii) **Preparation of Diets**: The 'control' diet consisted of:

121g 'Complan' (plain flavour)

98g Glucose (anhydrous)

10g Casein

5g (one sachet) 'Orovite-7'

- made up to one litre with cold tap water

The diet containing 20% of the total calories as ethanol consisted of:

121g 'Complan'

42g Glucose

10g Casein

5g 'Orovite-7'

32.5 mls Absolute alcohol

- made up to one litre as above
The diet containing 36% of the total calories as ethanol consisted of:

121g 'Complan'
10g Casein
5g 'Orovite-7'
56.5 mls Absolute alcohol

- made up to one litre as above

The diets were prepared in a Kenwood liquidiser to ensure uniform dissolution of the casein. All diets were prepared fresh daily. Diets were presented in graduated glass bottles with stainless steel nozzles. All bottles and nozzles were washed thoroughly daily, and were sterilised with a 1% (w/v) solution of sodium metabisulphite before fresh diet was placed in them. The composition of these alcohol-containing liquid diets, and the dietary regime are shown in Fig. 2.1.

**Pair-Feeding:** Ethanol-containing diets were placed in the drinking bottle so that each bottle contained 250 mls of diet, the level being marked with a waterproof pen. Next day, the volume of diet consumed by that animal (or pair of animals) was determined by counting the graduations in the bottle uncovered by the consumption of the diet. The average dietary intake of the ethanol consuming animals (mls/day) was calculated, and that volume of control diet was given to each animal (or pair of animals) in the corresponding control group. Thus, comparable calorie intakes between alcohol-fed rats and
Fig. 2.1.

COMPOSITION OF ALCOHOL-CONTAINING LIQUID DIET

DAYS 1 - 6
36% ETHANOL
20% CARBOHYDRATE
65% CARBOHYDRATE

DAYS 8 - 15
29% CARBOHYDRATE
19% FAT
16% PROTEIN

DAYS 16 - 36
19% FAT
16% PROTEIN

0% OF TOTAL CALORIES
control animals was maintained throughout. In some experiments, animals were housed individually, and in others, they were housed in pairs. The latter arrangement did not affect the average volume of diet consumed per animal, blood ethanol levels, or the severity of fatty liver induced.

(iii) Comparison of the 'Complan' diet with the Lieber-DeCarli diet:

A detailed comparison of the composition of these two diets is shown in Table 2.2. Some effects of the Complan diet on hepatic and serum parameters after chronic alcohol feeding are shown in comparison with reported values for the Lieber-DeCarli diet in Table 2.3. It will be seen that the composition of the Complan diet is not greatly different from the Lieber-DeCarli diet, although the former does have a lower proportion of calories present as fat, the percentage of calories arising from this source being closer to that found in normal rat solid diets than is the case for the Lieber-DeCarli diet. This in fact means that a higher proportion of calories are present as carbohydrate in the Complan diet, so possibly overcoming the objection of Rao and Larkin (1984) that carbohydrate deprivation during ethanol intake may produce fatty liver (see Section 1.3.7.). The concentration of some lipotropes and vitamins is lower in the Complan diet, but it appears that the latter is more palatable, and that animals consume slightly larger volumes of it per day than has been reported for the Lieber-DeCarli diet. Serum ethanol concentrations during ingestion of the Complan diet are also slightly higher as a result. The ethanol intake per unit body weight, body weight gains
TABLE 2.2

COMPARISON OF THE COMPOSITION OF THE 'COMPLAN' AND LIEBER-DECARLI ALCOHOL-CONTAINING LIQUID DIETS

<table>
<thead>
<tr>
<th>Component</th>
<th>'Complan Diet'</th>
<th>Lieber-DeCarli Diet*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calorie content</td>
<td>0.97 Kcal/ml</td>
<td>1.0 Kcal/ml</td>
</tr>
<tr>
<td>% total calories as:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>16.0</td>
<td>18.0</td>
</tr>
<tr>
<td>Fat</td>
<td>19.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Carbohydrate (Control diet)</td>
<td>65.0</td>
<td>47.0</td>
</tr>
<tr>
<td>Carbohydrate (36% ethanol</td>
<td>29.0</td>
<td>11.0</td>
</tr>
<tr>
<td>Choline</td>
<td>158 mg/l</td>
<td>250 mg/l</td>
</tr>
<tr>
<td>Methionine</td>
<td>948 mg/l</td>
<td>1500 mg/l</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>16.0 mg/l</td>
<td>30 mg/l</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>4000 U/l</td>
<td>approx. 6000 U/l</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>4.8 μg/l</td>
<td>10 μg/l</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>72 mg/l</td>
<td>N.S.</td>
</tr>
<tr>
<td>Vitamin B₁ (thiamine)</td>
<td>2.9 mg/l</td>
<td>0.73 mg/l</td>
</tr>
<tr>
<td>&quot; B₂ (riboflavin)</td>
<td>2.4 mg/l</td>
<td>1.25 mg/l</td>
</tr>
<tr>
<td>&quot; B₆ (pyridoxine)</td>
<td>2.5 mg/l</td>
<td>0.73 mg/l</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>3.6 mg/l</td>
<td>5.0 mg/l</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>25.7 mg/l</td>
<td>3.75 mg/l</td>
</tr>
</tbody>
</table>

*According to DeCarli and Lieber (1967)  N.S. = not stated
**TABLE 2.3**

**COMPARISON OF THE EFFECTS OF THE COMPLAN DIET AND LIEBER-DeCARLI DIET ON ETHANOL INTAKE AND HEPATIC PARAMETERS**

<table>
<thead>
<tr>
<th></th>
<th>COMPLAN</th>
<th>LIEBER-DeCARLI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Alcohol-Fed</td>
</tr>
<tr>
<td>Average diet intake* (ml/day)</td>
<td></td>
<td>65 - 90</td>
</tr>
<tr>
<td>Ethanol intake (g/kg/day)</td>
<td></td>
<td>12.8 - 15.1</td>
</tr>
<tr>
<td>Body wt. gain (g/week)*</td>
<td></td>
<td>19.9</td>
</tr>
<tr>
<td>Typical serum ethanol (mg/100 ml)</td>
<td></td>
<td>168 - 293</td>
</tr>
<tr>
<td>Total hepatic lipids (mg/g)</td>
<td>48.0 ± 10.7</td>
<td>90.7 ± 10.8</td>
</tr>
<tr>
<td>Hepatic triglycerides (mg/g)</td>
<td>10.2 ± 3.3</td>
<td>46.1 ± 13.3</td>
</tr>
</tbody>
</table>

Values for Complan diet are means or ranges of four complete experiments carried out with the diet, and represent at least forty animals per group. * = assessed during period of 36% calorie intake as ethanol. + From DeCarli and Lieber (1967).
and hepatic lipid changes after three weeks of ethanol intake with the Complan diet are almost identical to those that have been reported for a similar duration of alcohol intake with the Lieber-DeCarli diet (Table 2.3).

2.3 ASSAYS FOR LIVER LIPID CONTENT

Hepatic total lipid and triglycerides were assayed by the application of colorimetric serum methods to 12.5% (w/v) of liver homogenate. Dilution of liver homogenates was carried out using the same medium as was utilised for the original homogenisation step (ie: either isotonic saline or 0.25M sucrose).

2.3.1. Total lipid assay:

This was carried out using kits supplied by B.C.L. Ltd. The method is based upon the reaction of sulphovanillin with charred lipids in an acid medium to give a red colour (Woodman and Price, 1972). Aliquots (0.05 ml) of diluted homogenate or lipid standard (10 mg/ml in ethanol, as supplied with the B.C.L. kit) was placed in a clean test tube and 2.0 ml concentrated sulphuric acid added. The tubes were mixed and then plugged with cotton wool stops. They were heated in a boiling water bath for ten minutes, cooled in tap water, and 0.1ml aliquots of charred extract removed into clean test tubes. A blank was set up at this stage, consisting of 0.1 ml concentrated sulphuric acid. The colour reagent (2.5 ml of 13 mM vanillin in 14M phosphoric acid) was then added to all tubes, mixed, and the
absorbance at 530 nm read vs. the blank after 20-30 minutes. Accuracy of the method was checked using B.C.L. 'Preclip' quality control serum, and values were found to be within the quoted range for all analyses performed. Five replicate analyses of a liver homogenate prepared from a normal, solid-diet fed male Wistar rat gave a mean value of 35.3 mg/g liver, standard deviation being 1.75 mg/g, giving a coefficient of variation for the method of 4.9%. Coefficient of variation between duplicate analyses of rat liver homogenates was found to be 3.3%.

2.3.2. Liver Triglyceride Assay:

This assay was based on the colorimetric procedure described for serum by Fletcher (1968), and was carried out using kits supplied by Sigma Chemical Co. Lipids were extracted from the homogenate into isopropanol and all polar lipids absorbed onto activated alumina to leave the triglycerides in the solvent phase. After saponification, oxidation of released glycerol was carried out to yield formaldehyde, which was reacted with acetylacetone to give a yellow colour that absorbs at 405 nm.

Portions (0.2 ml) of diluted liver homogenate were placed in screw-cap test tubes containing 800 mg (+ 200 mg) activated alumina and 5.0 mls isopropanol. Blank tubes contained 0.2 ml saline in place of homogenate, and standards (0.75, 1.5, 2.25 and 3.0 mg triolein/ml in isopropanol) were set up at the same time. The standard solutions were added to tubes containing 4.8 ml isopropanol and 0.2 ml saline. All tubes were then capped, extracted for twenty
minutes on a rotary mixer, and then centrifuged at 1500 g for 10 minutes. An aliquot (2.0 ml) of the isopropanol phase was removed into a clean test tube and 0.5 ml 1N potassium hydroxide added. The tubes were mixed and heated in a 60°C water bath for five minutes, before cooling in tap water. Sodium-m-periodate (0.5 ml of a 2.5 mg/ml solution in 2N acetic acid) was then added to all tubes, a stop clock being started at the first addition, and the approximate time between additions noted. Exactly ten minutes after the addition of periodate, 3.0 ml colour reagent was added to all tubes at about the same rate as the periodate, so that all the tubes received colour reagent exactly ten minutes after the periodate addition. The colour reagent consisted of 20 ml 2M ammonium acetate added to 40 ml isopropanol and 0.15 ml acetylacetone. Best results are achieved if the reagent is allowed to 'age' overnight before use. The reagent is stable at 4°C in a dark bottle for seven days. After addition of colour reagent, the tubes were stopped with glass marbles and heated in a 60°C water bath for exactly thirty minutes. After cooling in tubes in tap water, absorbances were read against the blank at 405 nm within 20 minutes.

The calibration curve for the assay is shown in Fig. 2.1, and shows that the method obeys Beer's law. Accuracy assessment, using Preclip control serum, gave good results and five replicate analyses of a liver homogenate from a normal, solid diet-fed, male Wistar rat gave a mean liver triglyceride concentration of 7.0 mg/g with a standard deviation of 0.57 mg/g, so that the coefficient of variation
Fig. 2.2. CALIBRATION CURVE FOR TRIGLYCERIDE ASSAY
was 8.1%. Recovery studies using liver homogenate spiked with [1-\(^{14}\)C] triolein showed extraction efficiency in this method to be between 92 and 98%, overall recovery of unlabelled triolein added to homogenates being in the range of 88-92%.

2.4 LIVER METABOLITE ASSAYS

2.4.1. Sample collection:
Samples of tissue for metabolite analysis were collected by freeze-clamping. Animals were sacrificed by cervical dislocation, the abdomen rapidly opened, and a portion of the liver quickly frozen in situ using aluminium tongs which had been pre-cooled in liquid nitrogen. The frozen piece of tissue was excised, any portions protruding from the edges of the tongs broken off, and the frozen portion between the tongs stored in liquid nitrogen until extraction and analysis.

2.4.2. Sample extraction:
The frozen sample was ground to a powder under liquid nitrogen in a pestle and mortar. Approximately 800-1000 mg powdered tissue was placed in a pre-weighed homogeniser tube containing 5.0 mls ice-cold 0.6N perchloric acid, the mixture homogenised in the cold with a teflon pestle driven by an electric drill motor, and the homogeniser tube and contents then re-weighed to determine the exact amount of tissue added. The 'homogenate' was then centrifuged in the cold at 2000 g for ten minutes, the supernatent removed into a clean test tube
kept on ice, the residue in the centrifuge tube re-extracted with 2.0 ml 0.3N perchloric acid and centrifuged as above. The combined supernatents were then neutralised to pH 5-6 using 5M potassium carbonate with a pH meter. The clear extract, obtained after centrifuging away the potassium perchlorate precipitate was then treated with Florisil as described by Williamson et al (1967). Florisil (100-200 mesh) was then added (100 mg/ml extract), mixed, the extract centrifuged at 3000 g for 15-20 mins. The clear supernatent, free from interfering flavines as a result of the Florisil treatment, was then used for the metabolite assays as described below. The volume of this final extract was determined, and the mg liver tissue/ml extract calculated. ATP and pyruvate assays were carried out rapidly after preparation of the extract, as these substrates are the least stable in the neutralised preparation.

2.4.3. ATP assay:

ATP was measured by the enzymatic method originally described by Bucher (1947), using kits supplied by B.C.L. Ltd. All reagents were prepared as directed in the instructions of the kit. Triethanolamine buffer/glycerate-3-phosphate solution (2.0 ml), NADH (0.2 ml of a 2.5 mM solution), and 0.1 ml sample extract were placed in a spectrophotometer cuvette. After mixing, the absorbance at 340 nm was read against air at room temperature (reading A1). Enzyme suspension (0.2 ml; glycerate 1,3-diphosphate dehydrogenase/phosphoglycerate kinase/triosephosphate isomerase/glycerol phosphate dehydrogenase) was then added, and the
absorbance determined again after ten minutes, once the reaction had come to completion (= reading \( A_2 \)). The absorbance change (\( \Delta A \)) was then calculated:

\[
\Delta A = A_1 - A_2
\]

For each kit, the absorbance change due to addition of the enzyme suspension was determined by running the assay using water in place of the sample. \( \Delta A \) values were then corrected to allow for this.

2.4.4. Pyruvate Assay:

Pyruvate was measured by the method of Czok and Lamprecht (1974). Sample extract (2.0 ml) was placed in a cuvette containing 1.0 ml phosphate buffer (0.1M, pH = 6.8; prepared as described by Mellanby and Williamson (1974)) and 0.1 ml NADH solution (5 mg/ml in water). After mixing, and equilibration at room temperature, the absorbance at 340 nm was read against a cuvette containing 1.0 ml buffer and 2.1 ml distilled water (= reading \( A_1 \)). Lactate dehydrogenase (0.5 mg protein/ml; > 360 U/mg protein) suspension (0.01 ml) was then added, mixed, and the absorbance read again after five minutes. If the absorbance continued to decrease, further readings were taken at 10 and 15 minutes and the final absorbance determined by extrapolation to time zero to give reading \( A_2 \).

\[
\Delta A = A_1, - A_2.
\]

2.4.5. Acetoacetate Assay:

Acetoacetate was assayed as described by Mellanby and Williamson (1974), in the same cuvette as that used for the pyruvate assay (see above). After completion of the pyruvate assay, an absorbance
reading was taken \( (= A_3) \), and 0.01 ml 3-hydroxybutyrate dehydrogenase suspension added (5 mg protein/ml: BCL product was used undiluted). After 15-30 minutes, when the reaction had come to completion, a further reading was taken \( (A_4) \).

\[ \Delta A \text{ for the acetoacetate assay} = A_3 - A_4. \]

2.4.6. **Lactate Assay:**

Lactate was measured by the method of Gutmann and Wahlefeld (1974). Blank and sample cuvettes were set up as follows:

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>(0.4M Hydrazine, 0.5M glycine, pH = 9.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>(30 mg/ml in water)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample extract</td>
<td>-</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Perchloric acid (0.6N)</td>
<td>0.2 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

The buffer was prepared by dissolving 11.4 g glycine and 25 ml hydrazine hydrate (24%) in 200 ml water, checking the pH (should be 9.0), and making up to 300 ml with distilled water. The absorbance of the blank and sample cuvettes were read against air at 37°C at 340 nm. Lactate dehydrogenase suspension (0.01 ml of Sigma L-2500 undiluted) was then added to both cuvettes, mixed, and the cuvettes incubated at 37°C for 30 minutes. The absorbances were redetermined and the \( \Delta A \) values for the blank and sample cuvettes calculated. \( \Delta A \) for the calculation of results = \( \Delta A \) sample - \( \Delta A \) blank.
2.4.7. 3-Hydroxybutyrate Assay:

This substrate was assayed as described by Williamson and Mellanby (1974). Sample extract (1.0 ml) was placed in a cuvette containing 1.0 ml distilled water, 1.0 ml buffer and 0.1 ml NAD solution (10 mg/ml in distilled water) at room temperature. The buffer was prepared fresh daily by mixing 1 ml hydrazine hydrate (99%), 20 mg EDTA (disodium salt) and 5 ml 1N HCl, and diluting to 20 ml with Tris-HCl buffer (0.1M, pH = 8.5). Measurements were made at 340 nm against a blank cuvette containing 1.0 ml buffer and 2.0 ml distilled water. An initial absorbance reading was taken ($A_1$) and 0.01 ml 3-hydroxybutyrate dehydrogenase suspension (as for acetoacetate assay) added to both cuvettes. After one hour, the absorbance was read again ($A_2$) and the absorbance change calculated.

$$\Delta A = A_2 - A_1.$$ 

2.4.8. $\alpha$-Glycerophosphate Assay:

The method of Michal and Lang (1974) was employed for this metabolite. Sample extract (1.0 ml) was placed in a cuvette containing 1.0 ml buffer and 0.1 ml NAD solution (40 mg/ml in water). The buffer was prepared by suspending 5.2 g hydrazine sulphate, 7.5 g glycine and 0.2 g EDTA (disodium salt) in a little water, adding 51 ml 2N sodium hydroxide, and making up to a final volume of 100 ml with water. The pH was then checked (should be 9.5) and the buffer was discarded after one week. Measurements were made at room temperature against a water blank. Once a stable initial absorbance at 340 nm was achieved, reading $A_1$ was taken, and 0.01 ml $\alpha$-glycerophosphate dehydrogenase suspension (Sigma No. G6751 undiluted) added to the cuvettes. After mixing and waiting for the reaction to come to completion (about 10 minutes), reading $A_2$ was taken. $\Delta A = A_2 - A_1$. 

2.4.9. Calculation of Results from Metabolite Assays:

The concentration of metabolites per millilitre of tissue extract was calculated in the above assays using the following formula:

\[
\text{\( \mu \text{mol metabolite/ml extract} = \frac{\Delta A \times \text{Total assay volume (ml)}}{6.22 \times \text{Sample volume (ml)}} \)}
\]

\(6.22 =\) Molar extinction coefficient for NADH at 340 nm)

The above equation holds true providing that spectrophotometer cuvettes of 1 cm light path are used. The concentration of metabolite per g wet weight liver is then calculated by dividing the \( \mu \text{mol/ml extract} \) value by the mg tissue/ml extract value calculated earlier.

Some values obtained for various metabolites using the above methodology in fasted male Wistar rats are shown in Table 2.4, in comparison with published values obtained using identical methods. In experiments in which duplicate analyses of metabolite concentration were performed, the following coefficients of variation were obtained:

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>3.4%</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>12.1%</td>
</tr>
<tr>
<td>Lactate</td>
<td>5.6%</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>4.1%</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>2.9%</td>
</tr>
<tr>
<td>( \alpha )-Glycerophosphate</td>
<td>7.2%</td>
</tr>
</tbody>
</table>
## TABLE 2.4

**LIVER METABOLITE ASSAYS: VALUES OBTAINED USING METHODOLOGY DESCRIBED IN COMPARISON WITH REPORTED VALUES**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Values Obtained*</th>
<th>Reported Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>3726 ± 383</td>
<td>3250 ± 300+</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>42.4 ± 91.</td>
<td>47.0 ± 24.0++</td>
</tr>
<tr>
<td>Lactate</td>
<td>653 ± 195</td>
<td>780 ± 400+++</td>
</tr>
<tr>
<td>3-Hydroxybutyrate</td>
<td>1173 ± 69</td>
<td>1790 ± 830+++</td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>458 ± 33</td>
<td>500 ± 240+++</td>
</tr>
<tr>
<td>Lactate:Pyruvate</td>
<td>16.5 ± 8.0</td>
<td>17.0 ± 5.6+++</td>
</tr>
<tr>
<td>3-Hydroxybutyrate:Acetoacetate</td>
<td>2.58 ± 0.30</td>
<td>3.60 ± 1.4+++</td>
</tr>
<tr>
<td>α -glycerophosphate</td>
<td>142 ± 14</td>
<td>140 ± 20+++</td>
</tr>
</tbody>
</table>

*Values given were obtained in liver tissue from fasted male Wistar rats (mean of five animals)

+ From Lindros and Stowell (1982)

++ From Williamson et al (1967)

+++ From Michal and Lang (1974)

All values are given in nmol/g.liver (except ratios), and are shown as means ± S.D.
2.5 METHODS FOR INVESTIGATING HEPATIC LIPID UTILISATION AFTER ACUTE ETHANOL ADMINISTRATION

From the discussion in Chapter 1, it will be evident that ethanol metabolism causes disturbances in the normal lipid disposal of rat liver, that in turn could account for the production of fatty liver. Depressed fatty acid oxidation and increased esterification of fatty acids into triglycerides are the major features of this altered hepatic lipid metabolism. Thus, methods were set up to investigate the influence of the (+)-catechin compounds on these parameters after a moderate acute dose of ethanol (2.0 g/kg i.p).

The first experiment determined the hepatic uptake and incorporation of injected \([U^{14}C]\) palmitic acid into hepatic lipids after acute ethanol dosing, and assessing the effect of (+)-catechin in these respects. Secondly, the influence of the (+)-catechin compounds on ethanol-induced changes in the ability of liver tissue to oxidise \([1^{14}C]\) palmitate was investigated using liver slices taken from animals after acute in vivo ethanol administration.

2.5.1. Hepatic Uptake and Incorporation of \([U^{14}C]\) Palmitic Acid:

This method is based on that described by Abrams and Cooper (1976a), but incorporating some of the modifications outlined by Beauge et al (1979).

The label solution was prepared as follows. An appropriate aliquot of \([U^{14}C]\) palmitic acid in toluene was placed in a glass tube which had been pretreated with trichloromethylsilane (5% (w/v) in
chloroform), and evaporated to dryness under a stream of nitrogen gas at 37°C. Freshly-prepared rat serum from a fasted rat was then added to the tube so that the final concentration of radioactivity would be 10 μCi/ml serum. The tube was then vigourously vortex mixed and incubated at 37°C for 30 mins, and revortexed. The incubation and mixing was continued until a clear solution was obtained. This procedure resulted in 70-80% of the added radioactivity being bound to the rat serum. This value was found to be lower if serum from fed rats, or if non-silylated glassware was used. An aliquot of this preparation was solubolised in 0.5 ml Soluene-350 for 3-4 hours at room temperature, 10 mls Dimilume-30 liquid scintillant added, and the sample counted to determine the radioactivity concentration of this label solution.

Label solution (200 μl) was injected intraperitoneally at 90 mins after ethanol administration (2 g/kg i.p.). Label solution was prepared fresh and maintained at 37°C prior to injection. Exactly ten minutes after injection of label, the animal was sacrificed by cervical dislocation, and the liver rapidly excised into ice-cold 0.25M sucrose. After weighing, a 50% (w/v) homogenate was prepared in 0.25M sucrose using a Potter tube driven by an electric drill motor. Total radioactivity taken up by the liver was determined by solubilising 0.2 ml aliquots of homogenate in 1.0 ml soluene-350 at 55°C for three hours. The samples were then bleached by adding 0.2 ml hydrogen peroxide (30% v/v), and reheating at 55°C for one hour. Dimilume-30 (10 ml) was then added and the samples counted. The
percentage of the injected dose of [U-\(^{14}\)C] palmitate taken up by the liver was then calculated.

Lipids were extracted from the homogenate by the method of Folch et al. (1957). Homogenate (0.25 ml) was placed in a screw-cap sovriel tube (vol = 10 ml) and 4.25 ml chloroform:methanol (2:1) added. After capping, the tube was vortex mixed and shaken on a rotary mixer for 15 minutes. The mixture was then filtered through a Whatman No.1 filter paper which had been pre-moistened with chloroform:methanol (2:1) into a graduated, glass-stoppered pyrex tube (vol = 10 ml). The remaining residue in the sovriel tube was re-extracted briefly with 3.0 ml chloroform:methanol (2:1), using a vortex mixer, and then filtered through the same filter paper into the pyrex tube. The volume of the extract was adjusted to 7.5 ml in the pyrex tube with chloroform:methanol, 1.5 ml 0.88 (w/v) KCl added, and the tubes stoppered, and shaken on a rotary mixer for 5 mins before centrifuging at 2000 g for 10 mins to separate the phases. The upper phase was then aspirated as completely as possible without disturbing the lower phase. The lower phase was then washed three times by the careful addition of 1.0 ml aliquots of pure solvents upper phase (chloroform:methanol:water: 3:48:47). This was aspirated using a vacuum pump, and any remaining upper phase combined with the lower phase by the addition of methanol.

This extract was then taken to dryness under nitrogen gas at 37°C. The residue was redissolved in 0.5 ml chloroform:methanol (2:1), and 50 µl aliquots spotted out as 1 cm lanes on a silica gel 60
thin-layer chromatography plate. Appropriate markers were placed alongside the sample spots (triolein; 3 mg/ml in isopropanol; cholesteryl stearate; 1 mg/ml in isopropanol; lecithin; 1 mg/ml in methanol). The plates were developed in hexane:diethyl ether: acetic acid (80:20:1), and placed in a tank containing iodine vapour to visualise the spots. The areas of silica gel containing triglycerides, cholesterol esters and phospholipids were scraped from the plate into screw-cap test tubes, extracted with chloroform-methanol (1.0 ml), and aliquots counted in Dimilume-30 to determine radioactivity incorporated into individual lipid classes. An aliquot of the redissolved residue from the Folch extraction was also counted to give radioactivity incorporated into total liver lipids. Recovery studies using [1-\(^{14}\)C] triolein showed that 91% of the radioactivity added to liver homogenates was recovered after the Folch extraction and thin-layer chromatography, and that the coefficient of variation for recovered radioactivity between duplicate extractions and chromatographic separation was 9.7%.

2.5.2. Oxidation of [1-\(^{14}\)C] Palmitic Acid by Rat Liver Slices:

Animals were sacrificed 90 minutes after ethanol dosing, and livers rapidly excised into ice-cold Krebs-Hanseleit bicarbonate buffer (pH = 7.4). After thorough rinsing, a section of liver lobe was removed and sliced with a tissue grafting blade on a clean glass plate, moistened with buffer, and located on an ice bed. Slices were cut so that they were of the order of 0.3-0.5 mm thick.
Approximately 100-150 mg of slices (wet weight after blotting on Whatman No.1 filter paper) were placed in the outer well of a pre-weighed Ehrlenmeyer flask with a centre well, which contained 3.0 ml Krebs-Hanseleit buffer. The flasks were then reweighed to determine the exact amount of tissue added. Label solution (0.4 ml) containing [1-\textsuperscript{14}C] palmitic acid (2.5 \muCi/ml; 5 mM) was then added, septum caps placed in the flasks, the flasks sealed, and the preparations incubated for 90 minutes at 37°C in a shaking water bath (60 oscillations per minute). The reaction was stopped by injecting 0.5 ml 1M citric acid through the septum cap into the outer well. 0.6 ml Hyamine 10X solution was then injected into the centre well and the flasks further reincubated for 40 mins. After placing the flasks on ice for about 15 mins, the septum cap was then removed, and the contents of the well placed in 10 ml s Dimilume-30 liquid scintillation cocktail. The centre well was washed twice with 1.0 ml portions of scintillant, and the washings added to the counting vial. The slices and incubation medium were then homogenised using a Potter tube, and stored at -20°C until analysis for protein content and radioactivity incorporated into triglycerides. After counting, the radioactivity evolved as carbon dioxide, was corrected for quenching with an internal standard. The CO\textsubscript{2} production was expressed in either dpm/g liver or dpm/100 mg liver protein.

In experiments where total carbon dioxide production was measured, 0.5 ml barium hydroxide (0.3N) was placed in the centre well, after the incubation had been stopped with citric acid, and the
flasks left overnight at room temperature. The next day, 10 μl thymolphathlein indicator (0.1% (w/v) in ethanol) was placed in the centre-well and the contents titrated using 0.179N HCl dispensed through a Hamilton syringe (100 μl volume). The total CO₂ produced was calculated from the volume of acid required to neutralise the well contents, as described by Conway (1962). The specific activity of the carbon dioxide evolved from [1-¹⁴C] palmitic acid was then determined.

2.5.3. **Krebs-Hanseleit Bicarbonate Buffer (pH = 7.4, calcium free):**

This was prepared by mixing the following:

- 760 mls 0.9% (w/v) Sodium chloride
- 30.4 mls 1.15% (w/v) Potassium chloride
- 7.5 mls 2.11% (w/v) Potassium dihydrogen phosphate
- 7.5 mls 3.82% (w/v) MgSO₄·7H₂O
- 160 mls 1.3% (w/v) Sodium hydrogen carbonate

The pH was checked (should be 7.4) and adjusted if necessary with either 1N HCl or 1N NaOH. The volume was then made up to 1 litre with distilled water. The solution was bubbled with 100% O₂ for 10 mins, and the pH checked before use on each occasion.

2.5.4. **Label Solution ([1-¹⁴C] Palmitic Acid; 2.5 μCi/ml; 5 mM):**

An aliquot of [1-¹⁴C] palmitic acid label solution (in toluene) was placed in a silylated Sovriel tube and 100 μl of 18 mM unlabelled
palmitic acid solution (as sodium palmitate in methanol) added per 1 μCi of label dispensed. The contents of the tube were then dried down under a stream of nitrogen gas at 37°C. Freshly-prepared fatty acid-free bovine serum albumin solution (2% (w/v) in Krebs-Hanseleit buffer; 0.4 ml) was then added per 1 μCi of label, the tube vortex-mixed, and incubated at 37°C for one hour with occasional mixing, until a clear solution was obtained. This procedure resulted in greater than 90% of added radioactivity being bound to the serum albumin solution, providing fatty acid-free BSA and silylated Sovriel tubes were employed.

2.3.3. Other determinations:

Radioactivity incorporated into liver slice triglycerides during the incubations was determined as described above, for the incorporation of radioactivity into liver triglycerides after intraperitoneal injection of [U-14C] palmitic acid, but using 1.0 ml of homogenised incubation medium in place of 0.25 ml liver homogenate as the starting point for the Folch extraction procedure.

Liver slice protein content was determined by the method of Lowry et al (1951). Standard solutions of bovine serum albumin were prepared in distilled water (0.25, 0.50, 1.0 and 2.0 mg/ml). Aliquots of homogenised liver slice medium (20 μl) were placed in clean test tubes and made up to 0.5 ml with distilled water. Aliquots (0.5 ml) of standard solutions were likewise pipetted into test tubes, alongside a blank consisting of 0.5 ml of distilled water. Sodium hydroxide (1.0 ml of a 1.0N solution) was then added to all
Fig. 2.3. CALIBRATION CURVE FOR LOWRY PROTEIN ASSAY
tubes, mixed, and the tubes incubated at 37°C for 15 minutes. A 5.0 ml aliquot of freshly-prepared copper tartrate reagent (10 ml 1% (w/v) CuSO₄·5H₂O and 10 ml 2% (w/v) Na-K tartrate made up to 100 ml with distilled water) was then added to all tubes, mixed, and the tubes left to stand for 10 minutes. Folin-Ciocalteau reagent (0.5 ml; freshly-prepared by diluting 2N stock solution 1 in 2 just before use) was then added whilst each tube was being mixed on a vortex mixer. After 30 minutes the absorbances at 700 nm were read against the blank. A calibration curve was constructed, and protein content of the samples determined from this (see Fig. 2.3).

2.5.6. **Validation of fatty acid oxidation experiments:**

Initial experiments showed that recovery of radioactivity in the hyamine was dependent upon the presence of liver tissue in the outer wells of the flasks. Blank flasks, in which slices were omitted, showed negligible amounts of radioactivity recovered in the centre well at the end of the incubation. Using liver slices prepared from the same liver, it was found that ¹⁴CO₂ evolved was proportional to the amount of tissue in the incubation, up to a maximum of about 200 mg liver slices per flask. Above this amount, the yield of ¹⁴CO₂ per gram tissue decreased (Fig. 2.4). The evolution of ¹⁴CO₂ appeared to be linear up to 90 mins, the linearity disappearing after this time, hence 90 mins was used as the incubation time in all subsequent experiments (Fig. 2.5). The coefficient of variation for duplicate incubations (90 mins) of slices taken from the same liver was found to average 10.6% for all incubations carried out. In the first series
Fig. 2.4. EFFECT OF TISSUE WEIGHT ON $^{14}$CO$_2$ PRODUCTION FROM [1-$^{14}$C] PALMITATE BY RAT LIVER SLICES
Fig. 2.5. EFFECT OF INCUBATION TIME ON $^{14}$CO$_2$ PRODUCTION FROM [1-$^{14}$C] PALMITATE BY RAT LIVER SLICES

14CO$_2$ Production from 1-14C Palmitate (dpm/100mg liver protein x 10$^{-3}$) vs. Time (mins)

- 11.2±4.0
- 19.2±2.8
- 27.3±2.9
- 31.6±3.1
- 34.3±7.6
- 41.0±4.0
of experiments in which fatty acid oxidation by liver slices from ethanol-dosed animals was examined, no significant differences in ethanol-induced effects were observed when oxidation was expressed as dpm/90 mins/g liver as opposed to dpm/90 mins/100 mg liver protein. It is for this reason, that in later experiments, the protein determination was not carried out, and results expressed in relation to wet weight of tissue. When total CO₂ production was determined, it was found that ethanol dosing did not affect the specific activity of the evolved CO₂, indicating isotope dilution was not taking place, hence radiolabelled CO₂ evolution was a reliable indicator of CO₂ production in these experiments.

2.5.7. **Scintillation counting:**

All liquid scintillation counting was carried out using a Nuclear Enterprises NE 8312 radioactivity counter. Counting efficiency was determined by spiking samples with ¹⁴C-hexadecane liquid scintillation standard, and was found to be in the range 88-92% for all counting carried out in the above methods. All counts were corrected for efficiency, and expressed as disintegrations per minute (dpm).

2.6 **GAS CHROMATOGRAPHIC ANALYSIS OF ACETALDEHYDE AND ETHANOL IN RAT BLOOD, SERUM AND LIVER SAMPLES**

A method for the simultaneous measurement of acetaldehyde and ethanol in biological samples was devised, based on those described by
Eriksson *et al* (1977) and Von Wartburg and Ris (1979). Particular care was taken to ensure that true liver acetaldehyde levels were measured, and that artefactual formation of acetaldehyde from ethanol present in the samples was avoided, a problem encountered in many earlier studies (Korsten *et al*, 1975; Eriksson, 1980).

2.6.1. **Sample Collection and Preparation:**

Blood samples were obtained either from the necks of animals after sacrifice, or by closed cardiac puncture under ether anaesthesia. Liver samples were collected by freeze-clamping as described earlier for the determination of liver metabolites. Blood samples were allowed to clot, and then centrifuged to obtain the serum. Frozen liver samples were ground to a powder under liquid nitrogen (working in the cold room), and approximately 300 mg powdered tissue placed in a pre-weighed homogeniser tube containing 3.0 ml ice-cold 0.6N perchloric acid to which was added 25 mM thiourea to inhibit the artefactual formation of acetaldehyde from ethanol present in the sample. After brief homogenisation, the tube was reweighed to determine the exact amount of tissue added, and the contents immediately centrifuged for 5 mins at 3000 g at 4°C. A portion of the supernatent (2.0 ml) was immediately removed into a glass vial containing 1.0 ml *n*-propanol internal standard solution (0.5 mg/ml, freshly prepared) and 0.1 ml distilled water. The glass vial was capped with a teflon-lined rubber septum cap, and heated at 60°C in a water bath for exactly 30 minutes before 2.0 ml head-space was removed using a Pressure-Lok A2 gas syringe for gas chromatographic analysis.
Serum samples were treated as follows. Sample (50 µl) was placed in a glass vial containing 1.0 ml n-propanol solution as above, but the vial also contained 0.1 ml 60% (v/v) perchloric acid and 1.95 ml distilled water. The vial was sealed and heated for 30 mins at 60°C before removal of head-space samples, as described above for liver samples.

2.6.2. Gas Chromatography:

Analysis of head-space samples was carried out on a Hewlett-Packard 5710A Gas Chromatograph fitted with a 1.8 m x 4 mm I.D. glass column packed with Poropak-Q (50-80 mesh). Running conditions for the gas chromatograph were: column temperature 150°C; flame-ionisation detector temp: 250°C; injection port temperature: 200°C; nitrogen carrier gas flow: 30 ml/min., hydrogen flow: 30 ml/min., and air flow: 200 ml/min. The retention times for acetaldehyde, ethanol and n-propanol were 58, 98 and 260 seconds respectively. Calibration was carried out by treating aqueous solutions of acetaldehyde (2.0-200 nmol/ml) and ethanol (25-300 mg/100 ml; 5.4-65.2 mmol/l) in the same manner as the samples, peak height-ratios method being used to calculate the concentrations present in the samples. All standard solutions and the n-propanol internal standard solution were prepared fresh each day.

2.6.3. Validation of Methodology:

Construction of standard curves for the acetaldehyde and ethanol assays showed the detector response to be linear for both compounds (Figs. 2.6 and 2.7). The limit of detection for acetaldehyde was
Fig. 2.6. CALIBRATION CURVE FOR GAS CHROMATOGRAPHIC ACETALDEHYDE ASSAY
Fig. 2.7. CALIBRATION CURVE FOR GAS CHROMATOGRAPHIC ETHANOL ASSAY
approximately 75 pmol injected, which would allow measurement of blood or tissue acetaldehyde levels down to 1 μM. A chromatogram of a standard solution containing both acetaldehyde and ethanol is shown in Fig. 2.8. Extraction of frozen liver tissue into PCA-thiourea which had been spiked with 10 mM ethanol resulted in a low level of artefactual formation of acetaldehyde, this being equivalent to only 1.5 nmol/g liver at this ethanol concentration (Fig. 2.9). Artefactual formation of acetaldehyde was not enhanced at higher ethanol concentrations (up to 100 mM), but did become significant if heating of the extract at 60°C prior to removal of head-space samples was prolonged to greater than 45 minutes (results not shown). At an ethanol concentration of 80 mM, heating for 75 minutes instead of 30 minutes resulted in acetaldehyde equivalent to 20.9 nmol/g liver being formed artefactually, as opposed to only 1.5 nmol/g formed in the shorter incubation period. Artefactual formation problems could also arise if deproteinised liver samples were not immediately centrifuged after extraction of the tissue powder with PCA-thiourea. After homogenisation of rat liver powder in PCA-thiourea that contained 10 mM ethanol, only 1.5 nmol/g liver acetaldehyde was formed if the sample was immediately centrifuged and the supernatent removed (Fig. 2.9), whereas leaving the deproteinised sample on ice for one hour prior to centrifugation resulted in 8.7 nmol acetaldehyde/g liver being formed. Thus, in order to avoid artefactual formation problems, in assays for rat liver acetaldehyde, rapid centrifugation after the extraction procedure, and an incubation period at 60°C of
Fig. 2.8. GAS CHROMATOGRAM OF AN AQUEOUS SOLUTION CONTAINING 25 μmol/l ACETALDEHYDE AND 100 mg/100 ml ETHANOL
Fig. 2.9. GAS CHROMATOGRAM OF A RAT LIVER EXTRACT SPIKED WITH 10 mmol/l
(46 µg/g) ETHANOL
exactly 30 minutes were employed.

Recovery of 50 μM acetaldehyde added to PCA-thiourea in presence of rat liver powder, was found to be in the range of 94.1-104.5%. Duplicate extractions of portions of powdered tissue from the same rat liver after acute ethanol administration resulted in a coefficient of variation between the values obtained of 8.9%, at a mean liver acetaldehyde level of 24.7 nmol/g liver. This value was obtained in rat liver taken 90 mins after an intraperitoneal dose of ethanol (2.0 g/kg), and agreed well with published values for a similar ethanol dose at the same time point (Eriksson et al, 1983). Pre-treatment of animals with cyanamide, an aldehyde dehydrogenase inhibitor, (1 mg/kg p.o.) one hour prior to ethanol dosing (2 g/kg i.p), resulted in very high acetaldehyde levels being detectable in the liver (145.6-225.2 nmol/g liver).

Studies on the determination of blood and serum ethanol showed a mean recovery of 98.2% for human blood, 97.4% for human serum, 98.9% for rat blood and 97.9% for rat serum. The coefficients of variation for duplicate determinations were found to be below 2% for all these types of sample.

2.7 ENZYMATIC DETERMINATION OF ETHANOL IN BLOOD AND SERUM SAMPLES

In some experiments, ethanol was determined enzymatically using kits supplied by BCL Ltd (Cat. No. 123960). All reagents were prepared as directed in the instructions given with the kit. Samples
(50 μl of serum or blood) were mixed with 0.4 ml 0.33N perchloric acid (ice-cold), centrifuged, and 100 μl of supernatent used in the assay as instructed by the manufacturers. Aqueous standards were employed to calibrate the method, as for the gas chromatographic method. Analysis of six rat serum samples by both methods showed almost identical values achieved by each method, the correlation coefficient being 0.984 (P > 0.3). A calibration curve for the enzymatic method is shown in Fig. 2.10. One disadvantage of this method was that linearity of standard curve is lost above an ethanol concentration of 300 mg/100 ml, so it was not suitable for the measurement of very high blood or serum ethanol levels (data not shown). Coefficient of variation for duplicate analyses of serum samples using this method was found to be 1.3%.

2.8 ASSAYS FOR IN VITRO ANTIOXIDANT ACTIVITY OF DRUGS

2.8.1. Assay for Ability to Scavenge Superoxide Anions:

This procedure was based on the method described by Ponti et al (1978). NADH is used to reduce phenazine methosulphate which can then reduce molecular oxygen to produce superoxide anions. The rate of superoxide production can be determined in this system by following the reduction of nitroblue tetrazolium to a diformazan which absorbs at 560 nm. Superoxide scavenging ability of compounds is assessed by determining the degree of inhibition of diformazan formation when the drugs are added to the reaction mixture.
Fig. 2.10. CALIBRATION CURVE FOR ENZYMATIC ETHANOL ASSAY
(i) **Reagents:**

1. 0.1M Tris-HCl buffer (pH = 8.0).
2. 31.2 mM Phenazine methosulphate. Prepared fresh and kept protected from light.
4. 1.1 mM NADH. (2mg in 2.5 mls solution 1, prepared just before use).
5. Drug solutions were prepared either in water or dimethylformamide (DMF) at a concentration of 10 mM, so that addition of 30 μl to the incubation mixture would yield a final concentration of 100 μM, 15 μl would give 50 μM, etc.

(ii) **Method:** Distilled water (1.5 ml), 0.3 ml solution 1, 0.5 ml solution 3, and 0.2 ml solution 4 were placed in a spectrophotometer cuvette which was then positioned in a Cecil CE393 spectrophotometer at room temperature (λ = 560 nm). Drug solutions and/or water to a final addition volume of 30 μl were then added using micropipettes or a Hamilton syringe. A chart recorder was started, and once a stable baseline had been achieved, 0.5 ml solution 2 was added and mixed. The absorbance at 560 nm was recorded for 3-4 minutes and the mean initial change in absorbance per minute (ΔA/min) calculated from the chart trace.
(iii) **Results:**

<table>
<thead>
<tr>
<th>Addition to Reaction Mixture</th>
<th>ΔA&lt;sub&gt;560&lt;/sub&gt;/min</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.320 ± 0.005</td>
<td>-</td>
</tr>
<tr>
<td>10U Superoxide Dismutase</td>
<td>0.010 ± 0.002</td>
<td>96.9</td>
</tr>
</tbody>
</table>

(Sigma, from Bovine liver 3100 U/mg prot)

Values shown are the means ± S.D. of six determinations. The potent inhibitory action of superoxide dismutase demonstrated the specificity of this procedure for assessing superoxide scavenging ability of potential protective agents.

2.8.2. **Assay for ability to scavenge hydroxyl free radicals:**

This method was based on the ADP-chelated iron-NADPH system described by Slater (1968). Rat liver microsomes are incubated in the presence of ADP/Fe<sup>2+</sup>/NADPH and the formation of thiobarbituric acid (TBA)-reactive material determined after a 15 minute incubation, as an index of hydroxyl radical production and subsequent formation of lipid peroxidation products.

(i) **Reagents**

1. 0.22M Potassium chloride
2. 90 mM Tris-HCl buffer (pH = 8.0)
3. NADPH solution, 3.33 mg/ml in solution 2. (Prepared fresh)
4. ADP 10 mg/ml in solution 2. (Prepared fresh)
5. FeSO<sub>4</sub>·7H<sub>2</sub>O 40 μg/ml. (Prepared fresh)
6. 10% (w/v) Trichloroacetic acid
7. 0.67% (w/v) Thiobarbituric acid (TBA)
8. Drug solutions (10 mM in water or DMF). 30 μl aliquot added to the incubation gave a final concentration of 100 μM
(ii) **Method:** Washed microsomes were prepared from the livers of female Wistar albino rats (150-200 g) after an overnight fast by differential ultracentrifugation. The final preparation was suspended in 50 mM Tris-HCI (pH = 7.4) buffered saline (1.15% KCl), so that the microsomes from 1 g of liver were contained in 1 ml suspension.

Solution 1 (2.0 ml), 0.5 ml solution 2 and 0.1 microsome suspension were placed in a clean test tube. Appropriate aliquots of drug solutions (or water/DMF in the case of controls) were then added and mixed. Aliquots (0.1 ml) of solutions 3, 4, 5 were then added to each tube to start the incubation, and the tubes placed in a shaking water bath at 22°C for 15 mins. The incubation was stopped by removing 1.0 ml reaction mixture into a 10 ml sovriel tube containing 2.0 mls ice-cold solution 6. After mixing, the precipitated incubation mixtures were left on ice for 10-15 mins before centrifuging in the cold (30,000 g-min). After centrifugation, 2.0 ml clear supernatent was removed into a clean test tube and 2.0 mls reagent 7 added and mixed. The tubes were capped and heated in a boiling water bath for 10 mins, and then cooled in ice. The absorbance at 535 nm was then determined against a blank preparation, which consisted of microsomes taken through the above procedure, but with 0.1 ml aliquots of water being added in place of solutions 3, 4 and 5 at the beginning of the incubation. Results obtained using this method of determining hydroxyl radical scavenging activity are given in Chapter 4.
2.9 ASSAYS FOR HEPATIC LIPID PEROXIDATION

Two methods for measuring hepatic lipid peroxidation in vivo after acute ethanol administration were adopted. In view of the possible role of depressed glutathione concentrations in the development of hepatic lipid peroxidation after ethanol, an assay to measure the reduced form of this metabolite (GSH) was also set up.

2.9.1. Measurement of Hepatic Lipid Peroxidation by the Presence of Diene Conjugation in Microsomal and Mitochondrial Lipids:

Free radical attack of polyunsaturated fatty acids causes double bond rearrangement in these acids, so that conjugated dienes are formed as an intermediate in the peroxidative breakdown of the phospholipid fatty acids. These dienes absorb in the UV in the region 230-250 nm, whereas the polyunsaturated fatty acids, with their unconjugated double bond patterns, have peak absorbance at 210 nm. Thus, increased absorbance of a lipid extract in the 230-250 nm region, expressed per unit lipid, can be an indication of peroxidation having occurred. The method described for measuring diene conjugation has been adapted from that described by Hashimoto and Recknagel (1968).

(i) Method: Animals were sacrificed at various time intervals after the administration of ethanol (5 g/kg orally as a 20% (w/v) solution in water) or an equivalent volume of saline. The liver was rapidly excised into the ice-cold 10 mM potassium phosphate buffered 0.25M sucrose containing 3 mM EDTA (pH = 7.4). After rinsing, blotting and
weighing, the liver was minced with scissors and homogenised in sucrose-EDTA (as above) using a Potter tube driven by an electric drill motor to give a 50% (w/v) homogenate. This homogenate was then diluted to 10% (w/v) with sucrose-EDTA. This diluted homogenate was centrifuged at 400 g for 10 mins to sediment the nuclear pellet, and the supernatent was centrifuged at 400 g for 12 mins to produce a mitochondrial pellet. When microsomes were examined for diene conjugation, the 10% homogenate was spun at 7000 g for 20 mins to give a post-lysosomal supernatent which was then centrifuged at 100,000 g for one hour to produce a crude microsomal pellet. In the case of both mitochondria and microsomes, the quantities of original homogenate used were always such that the pellet obtained represented the subcellular fraction derived from 2.5 g of liver tissue.

(ii) Diene Conjugation Analysis: The supernatent above either the microsomal or mitochondrial pellet was decanted out of the centrifuge tube, after the preparation of subcellular fractions, and discarded. The tubes were then gently rinsed out with sucrose-EDTA, without disturbing the pellet, but removing any floating lipid that adhered to the wall of the tube after the centrifugation. Methanol (6.0 mls; Analar grade) was then placed in the tubes, vortexed to disperse the pellet, and the contents of the tube decanted into a 35 ml pyrex tube (acid-washed, glass stoppered). Chloroform (12.0 mls; Analar grade) was then added and the tubes allowed to stand at room temperature for 10 mins with occasional mixing. The tubes were then centrifuged for 5 mins at 400 g to sediment the insoluble material. The resulting
clear supernatant was carefully decanted into a clean, acid-washed, pyrex tube (35 ml, glass stoppered) and the volume of the extract made up to about 25.0 mls with chloroform:methanol (2:1). Distilled water (8.3 mls) was added to all tubes, and mixed gently by inversion. The tubes were then recentrifuged (400 g for ten minutes) to separate the phases, and the upper methanol-water layer was aspirated using a pasteur pipette attached to a vacuum pump. All the tubes were then left in ice for 5 mins, recentrifuged, and any remaining water-methanol droplets aspirated using the vacuum pump. An aliquot (2.0 mls) of the clear chloroform layer was then pipetted into a clean sovriel tube (10 ml, acid-washed), and evaporated to dryness under a stream of nitrogen gas at 40-50°C. After ensuring that all the chloroform was removed, the residue was redissolved in 3.0 mls cyclohexane (spectrosol grade, B.D.H). The absorbance of the cyclohexane extract was then read against a cyclohexane blank, in UV silica spectrophotometer cuvettes, at 300, 280, 260, 250, 245, 240, 235, 233, 232, 230, 225 and 220 nm. The cyclohexane extract was then retained, frozen at -20°C, for determination of its total lipid concentration.

The total lipid concentration was determined by the sulphovanillin method described earlier for liver homogenates, using kits supplied by B.C.L. Aliquots (0.5 ml) of the cyclohexane extract were placed in clean test tubes and evaporated to dryness under nitrogen gas at 40°C. Distilled water (50 μl) was added to each tube, and standards set up at this stage (50 μl aliquots of 10 mg/ml
lipid standard supplied with B.C.L. kit). Concentrated sulphuric acid (2.0 mls) was then added to all tubes, which were then plugged with cotton wool and heated in a boiling water bath for 10 mins. Determination of lipid concentration was then carried out exactly as described earlier (Section 2.3.1).

The lipid concentration in the cyclohexane extracts was calculated in mg.lipid/ml extract. All of the absorbances obtained in the diene conjugate analyses were then corrected to give absorbances for a 1 mg.lipid/ml extract. The corrected absorbances at each wavelength for each treatment group were plotted against wavelengths, and where appropriate, difference spectra (eg: ethanol-treated vs. saline controls) constructed. If lipid peroxidation had occurred in the subcellular fraction under study, this was revealed as a peak in the difference spectrum in the region 230-240 nm.

This method for determining diene conjugation was validated by studying liver microsomes isolated from rats 30 mins after an oral dose of carbon tetrachloride (2.5 mg/kg as a 1:1 solution in liquid paraffin). The absorbance of microsomal lipids from CCl₄-treated rats was found to be 0.525/mg.lipid at 233 nm compared to 0.375/mg.lipid at 233 nm in microsomal lipids isolated from control animals. These results gave a peak of 0.150 on the difference spectrum (CCl₄-treated rats vs. controls), this being almost identical to the change reported by Hashimoto and Recknagel (1968) after a similar dose of carbon tetrachloride, a substance well known to induce hepatic microsomal lipid peroxidation.
2.9.2. Measurement of Liver Malonaldehyde Levels by the Thiobarbituric Acid Reaction

Malonaldehyde (MDA), and its precursors, were determined using thiobarbituric acid as described for tissues by Uchiyama and Mihara (1978). Liver homogenates (10% w/v) were prepared as described for the diene conjugate analysis. A mixture of 0.5 ml homogenate, 1 ml thiobarbituric acid (0.6% (w/v) in water) and 3.0 mls of 1% (v/v) H$_3$PO$_4$ was incubated at 100°C for 45 mins in a boiling water bath. After cooling, the mixture was extracted with 4.0 mls butan-1-ol, the butanol phase separated by centrifugation (400 g for 10 mins), the upper phase removed, and its absorbance determined at both 535 nm and 520 nm against a butan-1-ol blank. The difference in absorbance at these two wavelengths was then calculated for each sample, and the malonaldehyde/malonaldehyde precursor concentration in the liver calculated assuming an absorbance coefficient of $1.56 \times 10^5$ mM cm$^{-1}$ for MDA. Coefficients of variation for this method of measuring MDA in liver tissue was 4.25% between duplicates. Analysis of control liver samples gave MDA values almost identical to those reported by Uchiyama and Mihara (1978).

2.9.3. Measurement of Liver Reduced Glutathione (GSH) Concentrations

This method is based on that described by Bernt and Bergmeyer (1974). A portion of 50% (w/v) liver homogenate, prepared as described in the diene conjugate method, was frozen in liquid nitrogen immediately after preparation and stored at -70°C until analysis.
Thawed homogenate (0.5 ml) was mixed thoroughly with 5.0 mls ice-cold 1.0N perchloric acid. After centrifuging away the protein precipitate, the supernatent was decanted into a graduated pyrex tube (10 ml), 1.0 ml 1.75M K₃PO₄ added and mixed. The pH of the extract was checked with a pH meter, and if necessary, further K₃PO₄ added to bring the pH to 6.5-7.0. The tube was then left on ice for a few minutes for the potassium perchlorate to settle out, and the volume of the extract was then determined so as to calculate the mg/liver/ml extract. The extract was then filtered through Whatman No.1 filter paper into a clean test tube. The following spectrophotometer cuvettes were then set up (UV silica cuvettes):

<table>
<thead>
<tr>
<th></th>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered Extract</td>
<td>0.5 ml</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>2.5 ml</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1% Egg Albumin</td>
<td>0.15 ml</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>Glyoxylase I</td>
<td>0.01 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

After mixing, the absorbance at 240 nm of the sample was read against the blank at room temperature (= reading A₁). A 0.02 ml aliquot of freshly-prepared methylglyoxal (0.1M) was then added to the sample cuvette, and the absorbance redetermined against the blank after five minutes (= reading A₂). Another 0.02 ml methylglyoxal was then added to the sample cuvette and the absorbance read again after one minute (= reading A₃)
\[ \Delta A = (A_2 - A_1) - (A_3 - A_2) \]

\[
\text{\textmu{}mol GSH/g.liver} = \frac{\Delta A \times 1}{3.37 \text{ mg.liver/ml in cuvette}}
\]

3.37 = Absorbance coefficient/\textmu{}mol/cm\(^2\) for S-lactoyl-GSH at 240 nm

Analysis of liver homogenates from control, fasted male rats gave a mean GSH concentration of 3.68 ± 0.82 \textmu{}mol/g.liver, a value close to those given in the literature. Coefficient of variation for the method was found to be 5.7% for duplicate analyses of the same liver.

2.10 MISCELLANEOUS ASSAYS

2.10.1. Serum Glutamate Dehydrogenase Activity (GLDH)

Serum glutamate dehydrogenase activity was determined by following the decrease in absorbance at 340 nm due to the oxidation of NADH and \(\alpha\)-ketoglutarate by the enzyme. All determinations were carried out using kits supplied by BCL Ltd. (Cat. No. 124320). The assay was carried out at 25°C according precisely to the instructions supplied with the kit. For each batch of samples run, the accuracy of the assay was checked using BCL 'Precinorm' quality control serum, all values obtained coming within the acceptable values quoted for this preparation. For rat serum, the coefficient of variation for duplicate analyses of the same sample was found to be 4.4%.
2.10.2. Statistical Analysis

Unless indicated otherwise, all experimental values given in the ensuing chapters are expressed as means ± standard deviation. Statistical significance of the difference between means values for various experimental groups was assessed by using Student's t-test for paired data. All statistical analyses were carried out by the author using an Apple computer. The following mathematical definitions were assumed in the programming process for the computer.

Mean (\( \bar{x} \)) = \( \frac{\sum x}{n} \)
where \( x \) = observed values
\( n \) = number of observed values

Standard deviation, \( s \), was determined according to the formula:

\[
s = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}
\]

where
\( \sum \) = 'sum of'
\( x \) = observed value
\( \bar{x} \) = arithmetic mean
\( n \) = number of observations
\( x - \bar{x} \) = deviation of a value from the mean, \( x \)
The 't' value in the Student's t-test for paired data was defined thus:

\[ t = \sqrt{n \cdot \frac{-\bar{d}}{s_d}} \]

where

- \( n \) = number of pairs of observations
- \( \bar{d} \) = mean of the differences between pairs of values
- \( s_d \) = standard deviation of the differences

The level of significance, expressed as a P-value, was determined by reference to statistical tables (Ciba-Geigy Scientific Tables)
Chapter 3

PROTECTIVE EFFECT OF (+)-CATECHIN COMPOUNDS AGAINST ACUTE AND CHRONIC ETHANOL-INDUCED FATTY LIVER

The aim of these initial experiments was to establish and confirm the hepatoprotective properties of (+)-catechin that have been described by previous authors (Gajdos et al, 1972). In the chronic alcohol feeding experiment, a comparison of the relative potency of (+)-catechin and 3-palmitoyl-(+) catechin at preventing fatty infiltration was carried out, so as to select the most suitable compound for further studies on the mechanism of action of these drugs.

3.1 METHODS

(i) Animals: Male Wistar Albino Rats (University of Surrey Strain, 180-220g) were used.

(ii) Ethanol Administration: Acute fatty liver was induced by administering ethanol as a single dose (6g/kg orally) in fasted rats (see Section 2.1). Chronic ethanol administration was carried out using the liquid diet formula described in Section 2.1. Ethanol feeding, as 36% of the total calorie intake, was carried out over a period of three weeks, following an introductory period to the diet, control animals being pair-fed diet in which the ethanol-derived energy was replaced by glucose.
(iii) Drug Administration: In the acute study, (+)-catechin was given as a suspension in saline (500 mg/kg i.p.) at 24, 3 and 0 hrs before ethanol. In one experiment, a single dose of catechin (500 mg/kg i.p.) was given 0.5h before ethanol.

In the chronic alcohol feeding study, (+)-catechin was given once daily (200 mg/kg orally) by gastric tube in 5% (v/v) Arabic Gum and 3-palmitoyl-(+)-catechin was given in a similar manner, but at a lower dose of 100 mg/kg orally. In all experiments, appropriate controls were sham-dosed with vehicle substances at the same time as the (+)-catechin compounds were administered.

(iv) Analyses: In the acute experiments, animals were sacrificed 16 hours after ethanol administration, livers removed, rinsed, blotted, weighed and homogenised in 0.25M sucrose. Hepatic triglycerides were determined as described in Section 2.2.

At the end of the chronic ethanol feeding experiment, animals were anaesthetised with diethyl ether, blood collected by closed cardiac puncture, and serum prepared. Animals were then sacrificed by cervical dislocation, and the liver removed, rinsed, blotted and weighed. A small piece of liver (\( \approx 200 \text{ mg} \)) was placed in formal-saline for histological examination using hemotoxylin and eosin staining. The remaining liver tissue was homogenised in 0.25M sucrose and analysed for total hepatic lipid and triglyceride contents. Serum was analysed for ethanol and GLDH activity.
TABLE 3.1

THE EFFECT OF (+)-CATECHIN ON THE ACUTE ETHANOL-INDUCED FATTY LIVER IN THE RAT

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic Triglyceride 16h after ethanol (mg/g liver)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>5.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Glucose + Catechin</td>
<td>5.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>20.9 ± 4.3*</td>
<td></td>
</tr>
<tr>
<td>Ethanol + Catechin</td>
<td>14.1 ± 1.8*</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>6.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Glucose + Catechin</td>
<td>6.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>19.6 ± 3.1*</td>
<td></td>
</tr>
<tr>
<td>Ethanol + Catechin</td>
<td>7.7 ± 2.9**</td>
<td></td>
</tr>
</tbody>
</table>

Fasted Male Wistar rats were given ethanol (6 g/kg p.o.) or an isocaloric dose of glucose (9.3 g/kg p.o.) and sacrificed 16 hours later. In experiment 1, a single dose of (+)-catechin (500 mg/kg i.p.) was given 0.5h before ethanol. In Experiment 2, three doses of (+)-catechin (500 mg/kg i.p. on each occasion) were given at 24, 3 and 0 hours before ethanol.

Values given are the means ± S.D. of six determinations.

* = p < 0.001 vs Glucose group.  † = p < 0.05 vs Ethanol only group.  ** = p < 0.001 vs Ethanol only group.
- 143 -

TABLE 3.2
THE EFFECT OF (+)-CATECHIN AND 3-PALMITOYL-(+)-CATECHIN ON CHRONIC ETHANOL-INDUCED FATTY LIVER IN THE RAT

<table>
<thead>
<tr>
<th>Parameters</th>
<th>A. Controls</th>
<th>B. Ethanol Only</th>
<th>C. Ethanol + Catechin</th>
<th>D. Ethanol + 3-Palmitoyl-(+)-Catechin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain during study (g)</td>
<td>86 ± 6</td>
<td>79 ± 8</td>
<td>84 ± 4</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>10.6 ± 1.0</td>
<td>10.0 ± 0.6</td>
<td>10.0 ± 0.8</td>
<td>9.8 ± 1.0</td>
</tr>
<tr>
<td>Total hepatic lipids (mg/g)</td>
<td>39.0 ± 8.9</td>
<td>153.0 ± 17.0*</td>
<td>99.0 ± 16.3**</td>
<td>76.0 ± 19.1*</td>
</tr>
<tr>
<td>Liver triglyceride (mg/g)</td>
<td>6.4 ± 2.1</td>
<td>59.0 ± 9.7*</td>
<td>34.5 ± 7.4**</td>
<td>29.0 ± 9.7**</td>
</tr>
<tr>
<td>Serum ethanol (mg/100 ml)</td>
<td>-</td>
<td>293 ± 59</td>
<td>275 ± 77</td>
<td>278 ± 81</td>
</tr>
<tr>
<td>Serum GLDH (U/l)</td>
<td>5.1 ± 1.3</td>
<td>15.8 ± 6.9*</td>
<td>6.6 ± 1.6**</td>
<td>8.7 ± 5.0</td>
</tr>
</tbody>
</table>

Ethanol was given as 36% of the total calorie intake over a period of three weeks in a liquid diet, controls receiving diet in which the ethanol energy was replaced by glucose. (+)-Catechin and 3-palmitoyl-(+)-catechin were given once daily throughout the feeding period at doses of 200 mg/kg orally and 100 mg/kg orally respectively. Values shown are the means ± S.D. of eight animals per treatment group.

* = p < 0.001 vs A.  ** = p < 0.001 vs B.  + = p < 0.001 vs B and p < 0.05 vs C.
3.2 RESULTS AND DISCUSSION

Data from the acute studies is shown in Table 3.1. A single dose of (+)-catechin (500 mg/kg i.p. 0.5h before ethanol) had only a partial protective effect against the acute alcoholic fatty liver, whereas divided doses of the drug (500 mg/kg i.p. at 24, 3 and 0 hours before ethanol) afforded almost complete protection against hepatic triglyceride accumulation. These findings parallel, to some extent, those of Slater and Scott (1981), who found that repeated high doses of (+)-catechin were necessary to prevent the acute carbon tetrachloride-induced fatty liver. Although not reported specifically in this chapter, subsequent experiments reported in this thesis (Chapters 5 and 9) showed that 3-palmitoyl-(+)-catechin, protects against the hepatic triglyceride accumulation after acute ethanol administration (either 2 g/kg i.p. or 5 g/kg p.o.).

Results from the chronic fatty liver study are shown in Table 3.2. Administration of (+)-catechin and 3-palmitoyl-(+)-catechin had no effect on liquid diet intake, and thus the ethanol intake expressed as g.consumed/kg body weight/day was almost identical between the ethanol-fed groups (data not shown). Neither compound influenced body weight gain or liver weights in the animals. Administration of ethanol alone caused 292%, 821% and 201% increases in the hepatic level of total lipid, triglycerides and the serum GLDH activity respectively. Administration of (+)-catechin reduced the extent of these increases to 153%, 439% and 29% respectively in ethanol-fed
rats, these effects of the drug being statistically significant. 3-Palmitoyl-(+)-catechin, even though it was given at only half the dose of the parent compound, was slightly more effective at preventing hepatic steatosis in ethanol-fed rats than (+)-catechin, the total hepatic lipid concentration in animals which received 3-palmitoyl-(+)-catechin being significantly less than that in the (+)-catechin-treated groups. The biochemical findings of reduced steatosis in the drug-treated animals were confirmed by histological assessment. The size of lipid vesicles was reduced, and the area of liver tissue affected lower in the animals treated with the (+)-catechin compounds (Fig. 3.1). Due to the wide individual variation in serum GLDH activities in the 3-palmitoyl-(+)-catechin treated animals, the decrease in this parameter in ethanol-fed rats caused by the drug was not statistically significant.

Serum ethanol concentrations at sacrifice were similar between all the ethanol-fed groups, suggesting that the drugs did not exert their hepatoprotective effects through interfering with ethanol absorption or elimination.

Pilot experiments, not reported in detail here, demonstrated that (+)-catechin also protected against the mild degree of fat accumulation induced by feeding 28% of the total calories as ethanol for 28 days. In order to compare the relative effectiveness of the two drugs at preventing fatty liver, it was decided to modify the liquid diet formula to contain a final level of 36% of the total calories as ethanol, as described in this thesis, in order to induce
more severe fatty infiltration of the liver. It was not possible, due to the large number of animals that would have been required, to assess the effects of the (+)-catechin compounds alone on the liver lipids in control animals in the same experiment as that reported here. However, a pilot experiment, in which (+)-catechin (200 mg/kg/day p.o.) was given to animals maintained on the control liquid diet for 36 days, showed the drug to have no influence on any of the biochemical or histological parameters used to assess the protective properties of the (+)-catechin compounds in the present experiment.

Thus, these studies have confirmed previous observations that (+)-catechin and, in addition, 3-palmitoyl-(+)-catechin, possess protective properties against ethanol hepatotoxicity in the rat (Gajdos et al, 1982; Perrissoud et al, 1985). The latter compound appeared to be more potent than (+)-catechin itself, in spite of the fact that it was given at only half the dose of the parent compound. In molar terms, allowing for the higher molecular weight of 3-palmitoyl-(+)-catechin (M.W. = 528) compared with (+)-catechin (M.W. = 290), the palmitoyl derivative is thus at least 3.6 times more effective at preventing ethanol-induced steatosis than (+)-catechin. Some possible reasons for this have already been indicated in the introductory chapter (see Section 1.5.2.), and probably include the greater lipid solubility and more favourable pharmacokinetic characteristics that result from the rate-limiting de-esterification step involved in the elimination of 3-palmitoyl-(+)-catechin (Hackett and Griffiths, 1982). Studies employing [U-\textsuperscript{14}C]-labelled
Fig. 3.1. **REPRESENTATIVE HISTOLOGICAL APPEARANCE OF LIVER TAKEN FROM RATS AT END OF CHRONIC ETHANOL-FEEDING STUDY**

Stain: Haematoxylin and Eosin (x 200)

Group A: Controls

Group B: Ethanol Only

contd. over ........
Group C: Ethanol and (+)-Catechin (200 mg/kg/day)

Group D: Ethanol and 3-Palmitoyl-(+)-Catechin (100 mg/kg/day)
(+)-catechin and [U-14C] 3-palmitoyl-(+)-catechin have shown that the half-life and bioavailability of the latter compound in the liver is much greater than for the parent compound after oral dosing (Ryle et al., 1983). The study of Hackett and Griffiths (1982) showed that perfusion of the isolated rat liver with 3-palmitoyl-(+)-catechin caused the release of (+)-catechin, and its glucuronide and sulphate conjugates, into the perfusate, indicating that the drug is converted to (+)-catechin after de-esterification in the liver. Thus, it might be assumed that the same molecule, namely (+)-catechin, is mediating the hepatoprotective effect of both compounds, 3-palmitoyl-(+)-catechin and (+)-catechin itself, after oral dosing. The mechanism of action of both drugs is probably similar at the biochemical level. Therefore, for many of the future studies on the mechanism of action of these drugs, 3-palmitoyl-(+)-catechin has been used, due to its greater potency, although it is probable that any findings regarding this compound's mode of action would also apply to the action of (+)-catechin itself.
Chapter 4

IN VITRO ANTIOXIDANT PROPERTIES OF (+)-CATECHIN COMPOUNDS

4.1 INTRODUCTION

The studies described in Chapter 3 have demonstrated the protective action of (+)-catechin and 3-palmitoyl- (+)- catechin against ethanol hepatotoxicity in the rat. As already discussed in the introductory chapter, there are probably two properties of these compounds that might account for these hepatoprotective effects. Firstly, there are the redox-state modulating effects of the compounds described by Gajdos et al (1972), and secondly, the free radical-scavenging (antioxidant) properties, described by Danni et al (1977) and Slater and Scott (1981), may afford the drugs these protective actions. The experiments in this chapter aim to confirm the antioxidant properties of (+)-catechin and 3-palmitoyl- (+)- catechin, and assess these relative to certain reference compounds known to possess antioxidant activity.

4.2 METHODS

The ability of substances to scavenge superoxide anions was assessed using the phenazine methosulphate-NADH-nitroblue tetrazolium method described by Ponti et al (1978), and given in detail in Section 2.8.1.
Hydroxyl radical scavenging was determined using the NADPH/Fe\(^{2+}\)/ADP system in rat liver microsomes, as described in detail in Section 2.8.2. Candidate drugs or reference compounds were dissolved in either water or dimethyl formamide (DMF), and added to the assay systems to give final concentrations in the range 1-100 μM. Depending upon the degree of inhibition of the radical-mediated effects in the assay systems, approximate EC\(_{50}\) values for each assay substance were calculated to obtain some impression of their relative effectiveness as antioxidants. The EC\(_{50}\) value was defined as the concentration of the scavenger in the assay system which achieved 50% inhibition of the radical-mediated action, when compared to the control incubations. In the case of the superoxide-scavenging assay, this value represents the concentration at which 50% inhibition of NBT reduction is achieved. In the case of the hydroxyl radical-scavenging assay, the value represents the concentration at which production of thiobarbituric acid-reactive material, produced after incubation of microsomes with NADPH/Fe\(^{2+}\), is inhibited by 50%.

Initial experiments showed that the solvent dimethylformamide had no effect on these parameters, when added to the incubations in amounts even in excess of those used to add potential scavengers to the assay systems.
4.3 RESULTS

Data on the ability of (+)-catechin and 3-palmitoyl- (+)-catechin to scavenge $O_2^-$ in the NADH/NBT/PMS assay are given in Table 4.1. Both compounds exerted an inhibitory effect on NBT reduction in this experiment. Data from the hydroxyl radical-scavenging studies are shown in Table 4.2. All of the compounds tested, including (+)-catechin and 3-palmitoyl(+) -catechin, exerted an inhibitory effect on the production of TBA-reactive material during incubation of rat liver microsomes with the hydroxyl radical generating system, NADPH/Fe$^{2+}$/ADP. The EC$_{50}$ values given in Table 4.3 indicate the relative effectiveness of the compounds tested at scavenging either superoxide or hydroxyl radicals. In the NADH/PMS/NBT system, (+)-catechin was twice as effective as 3-palmitoyl-(+)-catechin at scavenging superoxide anions. Conversely, in the NADPH/Fe$^{2+}$/ADP system, the more lipid-soluble 3-palmitoyl(+) -catechin appeared to be five times more effective than (+)-catechin itself at protecting rat liver microsomes against lipid peroxidation caused by hydroxyl radicals. In this respect, 3-palmitoyl-(+)-catechin was as effective as the reference synthetic antioxidants, DPPD (N,N'-diphenyl-p-phenylenediamine) and BHT (Butylated Hydroxytoluene), and slightly more effective than tocopherol acetate (vitamin E).
**TABLE 4.1**

**EFFECT OF (+)-CATECHIN AND 3-PALMITOYL-(+)-CATECHIN ON NBT REDUCTION IN THE PRESENCE OF PHENAZINE METHOSULPHATE-NADH**

<table>
<thead>
<tr>
<th>Addition to Incubation</th>
<th>$\Delta A_{560}$/min</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>$0.320 \pm 0.010$</td>
<td>-</td>
</tr>
<tr>
<td>IOU Superoxide dismutase</td>
<td>$0.010 \pm 0.001$</td>
<td>96.9</td>
</tr>
<tr>
<td>(+)-catechin (1 μM)</td>
<td>$0.316 \pm 0.007$</td>
<td>1.0</td>
</tr>
<tr>
<td>(+)-catechin (5 μM)</td>
<td>$0.302 \pm 0.012$</td>
<td>5.6</td>
</tr>
<tr>
<td>(+)-catechin (10 μM)</td>
<td>$0.295 \pm 0.006$</td>
<td>7.8</td>
</tr>
<tr>
<td>(+)-catechin (25 μM)</td>
<td>$0.239 \pm 0.010$</td>
<td>25.3</td>
</tr>
<tr>
<td>(+)-catechin (50 μM)</td>
<td>$0.172 \pm 0.008$</td>
<td>46.2</td>
</tr>
<tr>
<td>(+)-catechin (75 μM)</td>
<td>$0.090 \pm 0.004$</td>
<td>72.0</td>
</tr>
<tr>
<td>(+)-catechin (100 μM)</td>
<td>$0.022 \pm 0.002$</td>
<td>93.1</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (1 μM)</td>
<td>$0.323 \pm 0.011$</td>
<td>-</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (10 μM)</td>
<td>$0.310 \pm 0.009$</td>
<td>3.1</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (25 μM)</td>
<td>$0.287 \pm 0.006$</td>
<td>10.3</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (50 μM)</td>
<td>$0.252 \pm 0.005$</td>
<td>21.2</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (100 μM)</td>
<td>$0.172 \pm 0.018$</td>
<td>46.3</td>
</tr>
<tr>
<td>3-palmitoyl-(+)-catechin (200 μM)</td>
<td>$0.008 \pm 0.002$</td>
<td>97.4</td>
</tr>
</tbody>
</table>

Drug solutions were prepared in either water or dimethylformamide and added to the incubation mixture which contained Nitroblue Tetrazolium (NBT); 80 μM; NADH; 75 μM; Phenazine Methosulphate; 5μM; in Tris-HCl (pH = 8.0); 10 mM. NBT reduction was measured by following the change in absorbance at 560 nm. Values are shown as the means ± S.D. of at least three determinations.
**TABLE 4.2**

**EFFECT OF (+)-CATECHIN, 3-PALMITOYL-(+)-CATECHIN AND REFERENCE COMPOUNDS ON LIPID PEROXIDATION IN MICROSONES INCUBATED WITH NADPH/Fe²⁺/ADP**

<table>
<thead>
<tr>
<th>Addition to Incubation</th>
<th>A⁵³⁵/ml Incubation Medium</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0.348 ± 0.017</td>
<td>-</td>
</tr>
<tr>
<td>(+)-Catechin (0.1 µM)</td>
<td>0.340 ± 0.009</td>
<td>2.3</td>
</tr>
<tr>
<td>(+)-Catechin (1 µM)</td>
<td>0.313 ± 0.010</td>
<td>10.0</td>
</tr>
<tr>
<td>(+)-Catechin (10 µM)</td>
<td>0.023 ± 0.002</td>
<td>93.4</td>
</tr>
<tr>
<td>(+)-Catechin (100 µM)</td>
<td>0.017 ± 0.003</td>
<td>95.1</td>
</tr>
<tr>
<td>3-Palmitoyl-(+)-Catechin (0.1 µM)</td>
<td>0.330 ± 0.007</td>
<td>5.2</td>
</tr>
<tr>
<td>3-Palmitoyl-(+)-Catechin (1 µM)</td>
<td>0.179 ± 0.010</td>
<td>48.6</td>
</tr>
<tr>
<td>3-Palmitoyl-(+)-Catechin (10 µM)</td>
<td>0.010 ± 0.003</td>
<td>97.1</td>
</tr>
<tr>
<td>3-Palmitoyl-(+)-Catechin (100 µM)</td>
<td>0.002 ± 0.001</td>
<td>99.4</td>
</tr>
<tr>
<td>DPPD (0.1 µM)</td>
<td>0.331 ± 0.010</td>
<td>4.9</td>
</tr>
<tr>
<td>DPPD (1 µM)</td>
<td>0.231 ± 0.012</td>
<td>33.6</td>
</tr>
<tr>
<td>DPPD (10 µM)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>DPPD (100 µM)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>BHT (0.1 µM)</td>
<td>0.317 ± 0.009</td>
<td>9.0</td>
</tr>
<tr>
<td>BHT (1 µM)</td>
<td>0.207 ± 0.020</td>
<td>40.5</td>
</tr>
<tr>
<td>BHT (10 µM)</td>
<td>0.028 ± 0.005</td>
<td>92.0</td>
</tr>
<tr>
<td>BHT (100 µM)</td>
<td>0.020 ± 0.007</td>
<td>94.1</td>
</tr>
<tr>
<td>Tocopherol Acetate (1 µM)</td>
<td>0.282 ± 0.018</td>
<td>19.0</td>
</tr>
<tr>
<td>Tocopherol Acetate (10 µM)</td>
<td>0.057 ± 0.014</td>
<td>83.6</td>
</tr>
<tr>
<td>Tocopherol Acetate (50 µM)</td>
<td>0.019 ± 0.002</td>
<td>94.5</td>
</tr>
</tbody>
</table>

Rat liver microsomes were incubated for 15 mins. at 22°C with a hydroxyl radical generating system (NADPH/ADP/Fe²⁺) and the production of TBA-reactive substances determined after the incubation was stopped with TCA. Values shown are the means ± S.D. of at least three determinations. DPPD = N,N'-Diphenyl-p-phenylenediamine, BHT = Butylated Hydroxytoluene.
TABLE 4.3

EC$_{50}$ INHIBITORY CONSTANTS FOR VARIOUS COMPOUNDS IN THE SUPEROXIDE AND HYDROXYL RADICAL SCAVENGING ACTIVITY ASSAYS

<table>
<thead>
<tr>
<th>Substance</th>
<th>EC$_{50}$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O$_2^-$</td>
</tr>
<tr>
<td></td>
<td>(NADH/PMS/NBT)</td>
</tr>
<tr>
<td>(+)-Catechin</td>
<td>54 µM</td>
</tr>
<tr>
<td>3-Palmitoyl-(+) catechin</td>
<td>108 µM</td>
</tr>
<tr>
<td>DPPD</td>
<td>-</td>
</tr>
<tr>
<td>BHT</td>
<td>-</td>
</tr>
<tr>
<td>Tocopherol Acetate</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are calculated from the data given in Tables 4.1 and 4.2, and represent the concentration at which the radical-mediated end point event (NBT reduction, or production of TBA-reactive material) was inhibited by 50%. EC$_{50}$ values for DPPD, BHT and tocopherol acetate in the O$_2^-$ system were not determined due to turbidity problems in the assay.

DPPD = N,N'-Diphenyl-p-phenylenediamine
BHT = Butylated Hydroxytoluene
4.4 DISCUSSION

These experiments have demonstrated that (+)-catechin and 3-palmitoyl-(+)-catechin are potent scavengers of free radicals in vitro. With respect to superoxide scavenging, (+)-catechin is more effective than its palmityl derivative, possibly due to its greater solubility in water, and the fact that the NADH/PMS/NBT assay is in an aqueous environment. In the hydroxyl radical-scavenging system, however, 3-palmitoyl-(+)-catechin is more effective, probably as a result of its greater lipid solubility. In terms of protecting against free radical-mediated tissue damage in vivo, ability to scavenge hydroxyl radicals is probably more important than superoxide scavenging, since the former species is much more reactive and travels further within the cell from its site of production than does superoxide (Slater, 1984). In addition, there are ready-made mechanisms for detoxifying superoxide, through superoxide dismutase, in cells. Thus, in view of this, and also the pharmacokinetic factors discussed in the previous chapter, 3-palmitoyl-(+)-catechin is probably the best candidate to be an effective tissue antioxidant in vivo. Results from the hydroxyl radical-scavenging experiment (NADPH/Fe$^{2+}$/ADP system), and the EC$_{50}$ values calculated for the various scavengers, reveal that 3-palmitoyl-(+)-catechin is actually a slightly more effective scavenger than tocopherol (vitamin E), the substance thought to be the major lipid-soluble chain-breaking antioxidant in animal tissues (Slater, 1984).
These studies cannot accurately predict, however, the relative effectiveness of the substances tested at preventing lipid peroxidation in vivo. For a scavenger to be effective in vivo, it has got to be present at the site of free radical production at the right time, and in a suitable concentration. Thus, the ability of these substances to scavenge radicals in vivo depends, not only on their avidity for various radical species, but also on their absorption, distribution and elimination characteristics. Endogenous tocopherol, for example, is thought to reside in close proximity to arachidonic acid residues in membrane phospholipids, thereby protecting the molecule from free radical attack (Diplock and Lucy, 1973). However, in spite of its known antioxidant properties, tocopherol administration is not effective at preventing carbon tetrachloride hepatotoxicity in rats (Ghoshal, 1976). This has been attributed to inability of the administered vitamin to penetrate to the precise locus of generation of the \( \text{CCl}_3 \) radical in sufficient concentration to prevent this species causing membrane damage. Thus, in vivo antioxidant potency does not always correlate with in vitro findings. However, in view of the greater potency of 3-palmitoyl-(+)-catechin at scavenging hydroxyl radicals in vitro, and its pharmacokinetic and lipid solubility properties, the findings presented here suggest that this compound would be a much better candidate for preventing free radical-mediated tissue injury in vivo than (+)-catechin.
Chapter 5

EFFECTS OF (+)-CATECHIN COMPOUNDS ON THE HEPATIC
REDOX-STATE AND LIPID UTILISATION AFTER ACUTE ETHANOL
ADMINISTRATION

5.1 INTRODUCTION

The data described in the previous chapter has shown that the (+)-catechin compounds exert potent antioxidant properties in vitro, that might contribute to their protective actions against ethanol hepatotoxicity in vivo (Chapter 3). However, Gajdos et al (1972) suggested that (+)-catechin exerted its hepatoprotective effects through modulation of the disturbed NADH:NAD ratio observed after the administration of a number of hepatotoxins. The possible relationship between the increased NADH:NAD ratio arising from ethanol metabolism in the liver, the altered hepatic metabolism of fatty acids, and the development of alcoholic fatty liver has already been discussed in Chapter 1.

The aim of the work in this chapter was to re-examine the effect of (+)-catechin, in addition to 3-palmitoyl-(+)-catechin, on the hepatic redox-state after ethanol dosing, and to attempt to correlate any changes to the patterns of hepatic lipid disposal under the same experimental conditions.
5.2 METHODS

5.2.1. Animals and Administration of Drugs:

Male Wistar albino rats (University of Surrey strain, 180-220g) were used. They were maintained on laboratory chow and water and fasted for 24 hours prior to ethanol administration. (+)-Catechin and 3-palmitoyl(+)-catechin were given at doses of 200 mg/kg p.o. and 100 mg/kg p.o. respectively in 5% (w/v) Arabic Gum, at 24 and 0 hrs before ethanol dosing. Ethanol was given intraperitoneally (2 g/kg) as a 20% (w/v) solution in 0.9% NaCl. Controls were administered equivalent volumes of saline.

5.2.2. Redox-state and Fatty Acid Utilisation Studies:

(i) In vivo incorporation of [U-14C]-palmitic acid: [U-14C] Palmitic Acid was injected (i.p.) 90 mins. after ethanol in rat serum and ten minutes later, the animals sacrificed by cervical dislocation, and a portion of liver rapidly freeze-clamped in situ for metabolite determinations. The remainder of the liver was excised, rinsed in ice-cold 0.25M sucrose, blotted, weighed and homogenised in 0.25M sucrose. Incorporation of radioactivity into various hepatic lipid fractions was determined after extraction and separation by thin-layer chromatography (see Chapter 2, Section 2.5). The freeze-clamped liver sample was extracted with perchloric acid, and the lactate, pyruvate and α-glycerophosphate levels determined by standard enzymatic methods (Section 2.4). Hepatic triglycerides and serum ethanol were determined as already described (Section 2.3 and 2.7)
(ii) Oxidation of [1-\(^{14}\)C] palmitate by liver slices from ethanol-treated rats: Animals were sacrificed by cervical dislocation 90 mins. after ethanol-dosing and a portion of liver freeze-clamped in situ for metabolite determinations as described above. A further portion, well away from the freeze-clamping site, was removed into ice-cold saline, and cut into slices on an ice-bed using a tissue-grafting knife. Approximately 100-150 mg of slices were then incubated for 90 mins. in Krebs-Hanseleit bicarbonate buffer containing 1\(\mu\)Ci albumin-bound [1-\(^{14}\)C] palmitic acid. The incubations were stopped using 1M citric acid and the \(^{14}\)CO\(_2\) evolved during the incubation was collected into 10\% (w/v) hyamine 10X and counted. At the end of the incubations, the medium and slices were homogenised and protein content determined by the method of Lowry et al (1951). Full details of the methodology are given in Section 2.5.

5.3 RESULTS

The effect of (+)-catechin on the hepatic redox-state and incorporation of [U-\(^{14}\)C] palmitic acid into liver lipids after ethanol dosing is shown in Table 5.1. Ethanol caused a 220\% increase in the hepatic lactate/pyruvate ratio, due to both an increase in the lactate and a decrease in the pyruvate concentrations. Ethanol caused a 188\% increase in the hepatic \(\alpha\)-glycerophosphate concentration, and almost doubled the amount of injected radioactivity taken up by the liver and incorporated into the total hepatic lipids. Most of the latter was
TABLE 5.1
THE EFFECT OF (+)-CATECHIN ON THE HEPATIC REDOX-STATE AND INCORPORATION OF [U-14C] PALMITATE AFTER ACUTE ETHANOL ADMINISTRATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A Control</th>
<th>B Ethanol Only</th>
<th>C Ethanol + (+)-Catechin</th>
<th>D Catechin Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate (nmol/g.liver)</td>
<td>1080 ± 143</td>
<td>2262 ± 174*</td>
<td>1603 ± 305*</td>
<td>1097 ± 196</td>
</tr>
<tr>
<td>Pyruvate (nmol/g.liver)</td>
<td>168 ± 10</td>
<td>109 ± 10*</td>
<td>169 ± 11**</td>
<td>186 ± 15</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>6.5 ± 1.2</td>
<td>20.8 ± 1.6*</td>
<td>9.5 ± 1.9**</td>
<td>6.0 ± 1.3</td>
</tr>
<tr>
<td>⍵-Glycerophosphate (nmol/g.liver)</td>
<td>143 ± 10</td>
<td>412 ± 91*</td>
<td>366 ± 79</td>
<td>139 ± 32</td>
</tr>
<tr>
<td>% Injected [U-14C] palmitate in</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>liver lipids</td>
<td>8.6 ± 1.2</td>
<td>16.7 ± 1.6*</td>
<td>10.4 ± 1.9*</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>Total lipids</td>
<td>322 ± 37</td>
<td>794 ± 108*</td>
<td>373 ± 63**</td>
<td>351 ± 44</td>
</tr>
<tr>
<td>dpm x 10^-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides</td>
<td>97 ± 19</td>
<td>394 ± 30*</td>
<td>180 ± 30**</td>
<td>113 ± 19</td>
</tr>
<tr>
<td>incorporated/</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g.liver</td>
<td>161 ± 26</td>
<td>248 ± 49*</td>
<td>139 ± 40**</td>
<td>197 ± 22</td>
</tr>
<tr>
<td>Phospholipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol Ester</td>
<td>8.6 ± 3.6</td>
<td>26.8 ± 3.8*</td>
<td>10.5 ± 1.7**</td>
<td>4.9 ± 1.9</td>
</tr>
<tr>
<td>Triglyceride (mg/g liver)</td>
<td>3.54 ± 1.42</td>
<td>7.56 ± 2.54*</td>
<td>5.85 ± 2.08</td>
<td>3.36 ± 0.84</td>
</tr>
<tr>
<td>Serum Ethanol (mg/100 ml)</td>
<td>-</td>
<td>230 ± 19</td>
<td>210 ± 12</td>
<td>-</td>
</tr>
</tbody>
</table>

[U-14C] Palmitate (1 μCi/100 g b.w.) was injected 90 mins after ethanol (2 g/kg i.p.) and animals sacrificed 10 minutes later. (+)-Catechin was given (200 mg/kg p.o) at 24 and 0 hrs. before ethanol. Results are shown as means ± S.D. n = 5 rats per group. * = p < 0.01 vs. A. ** = p < 0.001 vs. A. + = p < 0.02 vs. B. ++ = p < 0.001 vs. B.
accounted for by a 300% increase in radioactivity incorporated into
the triglyceride fraction, although significant increases in
radioactivity incorporated into phospholipid and cholesterol esters
were also seen. The hepatic triglyceride concentration was doubled
by ethanol administration. Pre-treatment of ethanol-dosed animals
with (+)-catechin almost completely reversed all of these
ethanol-induced effects. A significant reduction in the
lactate/pyruvate ratio due to drug administration was found, mainly as
a result of increased pyruvate levels. Interestingly,
α-glycerophosphate levels were not significantly reduced by
(+)-catechin.

Nearly all of the ethanol-induced alterations in hepatic disposal
of [U-14C] palmitate were counteracted by (+)-catechin, significant
decreases in the incorporation of radioactivity into all the lipid
fractions being seen. (+)-Catechin also decreased the hepatic
triglyceride level in ethanol-dosed animals, although this effect was
not statistically significant due to the wide individual variation.
Serum ethanol levels indicated that (+)-catechin did not achieve these
effects simply through inhibition of ethanol oxidation. (+)-Catechin
alone did not significantly affect any of the parameters studied. A
diagramatic representation of the hepatic fatty acid disposal patterns
in the various treatment groups is given in Fig. 5.1.

The results of the experiments in which the effects of
(+)-catechin and 3-palmitoyl(+)-catechin on the hepatic redox-state
and palmitate oxidation after ethanol dosing were studied are shown in
Table 5.2. As found in the previous experiment (Table 5.1),
Fig. 5.1. EFFECT OF (+)-CATECHIN ON HEPATIC INCORPORATION OF [U-14C] PALMITATE INTO LIVER LIPIDS AFTER ACUTE ETHANOL ADMINISTRATION.

Animals were sacrificed 100 mins. after ethanol (2 g/kg i.p.). (+)- Catechin (200 mg/kg p.o.) being given at 24 and 0 hours before ethanol. [U-14C] palmitate (1μCi/100 g b.w.) was injected i.p. 10 mins. before sacrifice.
### TABLE 5.2

**THE EFFECTS OF (+)-CATECHIN AND 3-PALMITOYL-(+)-CATECHIN ON THE HEPATIC REDOX-STATE AND PRODUCTION OF $^{14}$CO$_2$ FROM [1-$^{14}$C] PALMITATE BY LIVER SLICES AFTER ACUTE ETHANOL ADMINISTRATION**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A Control</th>
<th>B Ethanol Only</th>
<th>C Ethanol + (+)-Catechin</th>
<th>D Ethanol + 3-Palmitoyl-(-)-Catechin</th>
<th>E (+)-Catechin Only</th>
<th>F 3-Palmitoyl- (+)-Catechin Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{14}$CO$_2$ released from liver slices</td>
<td>265 ± 39</td>
<td>98 ± 13*</td>
<td>197 ± 40**</td>
<td>248 ± 28**</td>
<td>278 ± 29</td>
<td>291 ± 35</td>
</tr>
<tr>
<td>(dpm x 10$^{-2}$/100 mg protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate (nmol/g.liver)</td>
<td>918 ± 76</td>
<td>1748 ± 70*</td>
<td>1323 ± 86**</td>
<td>1104 ± 72**</td>
<td>1002 ± 40</td>
<td>928 ± 101</td>
</tr>
<tr>
<td>Pyruvate (nmol/g.liver)</td>
<td>167 ± 13</td>
<td>112 ± 10*</td>
<td>157 ± 14**</td>
<td>178 ± 14**</td>
<td>170 ± 12</td>
<td>181 ± 19</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>5.7 ± 0.5</td>
<td>15.7 ± 1.3*</td>
<td>8.6 ± 1.2**</td>
<td>6.2 ± 0.4**</td>
<td>5.9 ± 0.9</td>
<td>5.1 ± 0.7</td>
</tr>
<tr>
<td>$\alpha$-Glycerophosphate (nmol/g.liver)</td>
<td>142 ± 14</td>
<td>486 ± 83**</td>
<td>404 ± 44</td>
<td>367 ± 61†</td>
<td>139 ± 17</td>
<td>133 ± 16</td>
</tr>
<tr>
<td>Serum Ethanol (mg/100 ml)</td>
<td>-</td>
<td>213 ± 29</td>
<td>192 ± 24</td>
<td>187 ± 30</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Animals were sacrificed 90 mins. after ethanol (2 g/kg/ i.p.), a portion of liver freeze-clamped for metabolite determinations, and liver slices prepared which were then incubated for 90 mins. in Krebs-Hanseleit buffer containing 1 µCi albumin-bound [1-14C] palmitic acid. (+)-Catechin and 3-palmitoyl-(+)-catechin were given at doses of 200 mg/kg and 100 mg/kg respectively (p.o.) at 24 and 0 hrs. before ethanol. Results are shown as means ± S.D. $n = 5$ rats per group. * = $p < 0.001$ vs. A. ** = $p < 0.001$ vs. B. † = $p < 0.005$ vs. B.
ethanol caused increases in the hepatic lactate/pyruvate ratio and α-glycerophosphate concentrations. Ethanol dosing caused a 62% decrease in the ability of liver slices to oxidise [1-14C] palmitate to 14CO2. (+)-Catechin partly, and 3-palmitoyl-(+)-catechin completely, prevented this decrease in fatty acid oxidation, at the same time as preventing the ethanol-induced hepatic redox-state changes, as already described for (+)-catechin in the previous experiment (Table 5.1). With respect to the comparative ability of (+)-catechin and 3-palmitoyl-catechin to reverse the ethanol-induced hepatic changes, the latter compound, in spite of being given at a lower dose than that of the parent drug, appeared to be slightly more effective than (+)-catechin itself at correcting both the redox-state change and the decreased fatty acid oxidation. As previously, neither (+)-catechin compound had any significant effect on any of the parameters studied when administered alone, and the drugs did not influence serum ethanol concentrations in ethanol-dosed rats.

5.4 DISCUSSION

These experiments have confirmed the findings of Gajdos et al (1972) that (+)-catechin is able to correct the altered hepatic redox-state (NADH:NAD ratio) resulting from ethanol administration, and shown that 3-palmitoyl-(+)-catechin also exerts this effect. 3-Palmitoyl-(+)-catechin is, in fact slightly more effective than (+)-catechin in this respect, in spite of the lower dose employed.
As was discussed in the previous chapter, this is probably due to its more favourable pharmacokinetic characteristics. In this experiment, only the lactate/pyruvate ratio was studied, being an indicator of the cytosolic NADH:NAD ratio, although it is possible that the drug also reverses disturbances in the mitochondrial redox-state as well. It is thought that the nicotinamide adenine dinucleotides do equilibrate between the two cell compartments, through the various shuttle systems involved in NADH transport into the mitochondria, so that a change in the cytosolic redox-state will be reflected in the mitochondria (Baroana and Lieber, 1979). Subsequent studies reported in this thesis show that correction of the hepatic redox-state changes after ethanol administration by Naloxone involves normalisation of both the cytosolic and mitochondrial redox-states (Chapter 6).

It is not clear from these experiments exactly how the (+)-catechin compounds achieve their correction of the redox-state changes. Gajdos et al (1972) proposed that (+)-catechin may stimulate glycolysis, generating pyruvate to reoxidise NADH by way of the lactate dehydrogenase reaction. Some of the present results support this view, since (+)-catechin did not fully restore \( \alpha \)-glycerophosphate levels to normal in ethanol-treated animals, thus possibly indicating that concentrations of the glycolytic intermediate dihydroxyacetone phosphate may be increased, this resulting in elevated \( \alpha \)-glycerophosphate levels, even though the redox-state had been corrected. Conversely, if (+)-catechin influences the redox-state through stimulating pyruvate production, one might expect
to see, if not increased pyruvate, then definitely increased lactate levels, over and above those seen in animals given ethanol alone, when (+)-catechin is given with ethanol. This was not found to be the case, however, so some doubt still surrounds this possible mechanism of redox-state modulation by the drug.

Alternative mechanisms for the correction of redox-state changes by (+)-catechin include direct, non-enzymatic oxidation of NADH, as is carried out by hydrogen acceptors such as Methylene Blue, or increased NADH oxidation in the mitochondria. The latter mechanism is a possibility, since bioflavonoids have been observed to have an uncoupling effect on oxidative phosphorylation in isolated mitochondria (Gajdos et al, 1972). Both of these mechanisms seem a little unlikely, since they might result in greatly decreased hepatic ATP levels, whereas (+)-catechin has in fact been reported to increase hepatic ATP concentrations (Gajdos et al, 1972). Serum ethanol measurements made in these experiments indicate that (+)-catechin does not prevent the ethanol-induced redox-state changes and effects on lipid disposal through inhibition of ethanol oxidation, as is the case for the alcohol dehydrogenase inhibitor pyrazole (Blomstrand et al, 1973).

The (+)-catechin compounds also reversed nearly all of the ethanol-induced alterations in hepatic fatty acid disposal in these experiments. Ethanol caused a significant increase in hepatic triglyceride, in association with increased palmitate esterification in vivo, and decreased palmitate oxidation by liver slices in vitro,
these latter changes possibly accounting for the hepatic lipid accumulation. It could be assumed that reversal of these abnormalities in lipid metabolism by the (+)-catechin compounds is the mechanism by which the compounds lower the extent of the ethanol-induced hepatic triglyceride accumulation already described (Chapter 3). Inhibition of triglyceride accumulation in the present study was however, not statistically significant due to the wide individual variation in the animals studied.

The main question that is posed by these results, is whether the reversal of the fatty acid disposal alterations after ethanol administration by (+)-catechin is the direct result of correction of the redox-state (NAD/NADH ratio), along with the implications of this for intermediary metabolism as already discussed (Section 1.2.3.). It is not clear whether other substances which prevent ethanol-induced hepatic triglyceride accumulation in the acute situation also affect fatty acid utilisation in the same way as which (+)-catechin does, and whether they also mediate these effects through modulation of the hepatic redox-state. It should be remembered that, in the present experiments, decreased fatty acid oxidation was found in liver tissue taken from ethanol-dosed animals, no ethanol being added to the incubation medium. Thus, this alteration in lipid disposal may not be the consequence of direct, acute actions of ethanol (ie: redox-state changes) in the liver. In order to resolve these problems, in the succeeding chapter, the effect of sorbitol administration on hepatic lipid disposal has been studied, since this
substance also induces a redox-state change. In addition, the effect of correcting the hepatic redox-state change after ethanol dosing with Naloxone, as reported by Badawy and Evans (1981) and Badawy and Aliyu (1984), on the various parameters studied here, has been determined (Chapter 6). These studies should clarify the true relationship between redox-state changes and the triglyceride accumulation after ethanol dosing, and indicate the importance, or otherwise, of (+)-catechin's effects on the redox-state in the mechanism of action of this drug.

In view of the antioxidant properties of the (+)-catechin compounds described in Chapter 4, the influence of synthetic antioxidants on fatty liver production has been determined (Chapter 7).
Chapter 6

MODULATION OF THE REDOX-STATE: INFLUENCE ON ACUTE AND CHRONIC ETHANOL-INDUCED HEPATIC LIPID ACCUMULATION

6.1 INTRODUCTION

Two approaches have been adopted here to attempt to answer the question posed at the end of the previous chapter, as to whether correction of the redox-state by the (+)-catechin compounds is responsible for the changes in hepatic lipid disposal after ethanol dosing. Firstly, the effect of acute sorbitol dosing on the hepatic redox-state and fatty acid utilisation has been determined, since this carbohydrate elicits a redox-state change, similar to that caused by ethanol, through its metabolism by sorbitol dehydrogenase. A dose of sorbitol, isocaloric to the ethanol dose employed in the previous experiments, was used and glucose-dosed animals served as controls.

Secondly, the influence of the opiate antagonist, Naloxone, on hepatic lipid utilisation after acute ethanol administration has been investigated. This drug is known to correct the ethanol-induced redox-state change, without inhibiting ethanol metabolism (Badawy and Evans, 1981; Badawy and Aliyu, 1984). These experiments were designed to give information on the relationship between the redox-state changes and altered hepatic lipid disposal after acute ethanol administration. Since data obtained on fatty acid oxidation
by liver slices taken after ethanol dosing may not be a true representation of events occurring in vivo, as sorbitol or Naloxone were not added to the incubation medium in these experiments, some in vitro studies were also carried out. These compared the effects of sorbitol and ethanol, added to liver slice incubations, on fatty acid oxidation and esterification, and related these parameters to redox-state changes in the in vitro assay system.

These acute studies should indicate the role of the (+)-catechin compounds' effects on the redox-state in preventing the acute ethanol-induced lipid accumulation, as already described. In order to establish whether these properties of (+)-catechin and 3-palmitoyl-(+)-catechin might afford protection against fatty liver in the chronic ethanol-feeding model, an attempt has also been made to modify the redox-state under the latter experimental conditions. This was achieved by observing the influence that Methylene Blue has on the development of fatty liver after chronic ethanol feeding.

Methylene Blue is a hydrogen acceptor which carries out non-enzymatic oxidation of NADH and NADPH in vivo, so that some degree of correction of redox-state changes during ethanol ingestion can be achieved (Hrushesky et al, 1985). An attempt to assess the influence of Methylene Blue, as well as another hydrogen acceptor, Menadione, on chronic alcoholic fatty liver was made by Lieber and DeCarli (1966) in their study on hepatoprotective agents. They found that neither compound had any protective effect against hepatic steatosis, although no redox-state measurements were made, and the effects of Methylene
Blue alone on hepatic lipids were not fully reported. Thus, in the present experiments, the Lieber and DeCarli (1966) study has been repeated, using a similar level of supplementation of the alcohol-containing liquid diet with Methylene Blue, but, in addition, redox-state measurements have been made, and an appropriate, pair-fed, Methylene Blue-treated control group employed. One of the possible consequences of the mechanism of action of Methylene Blue, is that non-enzymatic oxidation of NADH and NADPH may cause a short-fall in ATP synthesis in the mitochondria, as well as possible affecting reduced glutathione concentrations, due to a decrease in NADPH to act as a cofactor for glutathione reductase. Thus, both ATP and reduced glutathione (GSH) concentrations have been measured in this study to determine whether any effects of Methylene Blue on these parameters might affect hepatic function, and the susceptibility of the animals to hepatic damage caused by ethanol.

6.2 METHODS

6.2.1. Acute in vivo studies:

Male Wistar Albino rats (University of Surrey strain, 180-220 g) were used. They were starved for 24 hours prior to receiving either ethanol (2 g/kg i.p. as a 20% (w/v) solution) or isocaloric sorbitol (3.5 g/kg i.p. as a 35% (w/v) solution). Saline served as the control substance in the case of ethanol-dosed animals, whereas glucose was used as a control in the sorbitol experiments. Naloxone
hydrochloride was dissolved fresh in sterile saline and given 1.0 hour, and, where appropriate 2.5 hrs., after ethanol administration (2 mg/kg i.p.). Ninety minutes after administration of either ethanol or sorbitol, animals were injected with [U-\textsuperscript{14}C] palmitic acid (1 \mu Ci/100 g b.w.) and killed 10 mins. later for the determination of radioactivity incorporated into hepatic lipids, as described previously (Section 5.2). Alternatively, animals were sacrificed at 90 mins. for the preparation of liver slices which were then incubated for 90 mins. in Krebs-Hanseleit buffer (pH = 7.4) containing 1 \mu Ci albumin-bound [l-\textsuperscript{14}C] palmitic acid, as described in Section 2.5.2. Freeze-clamped liver samples were obtained at sacrifice for the determination of the hepatic lactate, pyruvate, \alpha-glycerophosphate, 3-hydroxybutyrate and acetoacetate concentrations as described previously. ATP was assayed using kits supplied by BCL Ltd. Hepatic triglycerides were measured by the method of Fletcher (1968), as already described (Section 2.3.2.), at 1.5h and, in the Naloxone studies, also at 3.0h after ethanol dosing.

6.2.2. In vitro studies:

Liver slices were prepared from male Wistar albino rats after an overnight fast, and incubated in Krebs-Hanseleit bicarbonate buffer (pH = 7.4) containing 1 \mu Ci albumin-bound [l-\textsuperscript{14}C] palmitate as described previously (Section 2.5.2.). Glucose, fructose, sorbitol or ethanol were added to the incubation medium at concentrations indicated on the Results tables. At one hour after the start of the incubation, 1.0 ml of incubation medium was removed through the septum
cap of the incubation tube, using a disposable syringe, immediately mixed with 1.0 ml ice-cold 1N perchloric acid, and centrifuged in the cold. Aliquots of the supernatent were used for the assay of lactate and pyruvate using kits supplied by BCL Ltd. (cat. no. 124842 and 124982 respectively). The incubation was stopped with 1M citric acid, and $^{14}$CO$_2$ evolved determined as described previously (Section 2.3.2.). The incubation medium was homogenised, and a 1.0 ml portion of 'homogenate' was extracted with 5.0 mls chloroform:methanol (2:1) on a roller mixer for 20 mins., and the extract filtered through Whatman No.1 filter paper. The residue was then extracted with 2.0 mls chloroform:methanol (2:1), filtered, and this second extract combined with the first one. The Folch washing step was then carried out (Section 2.3.1.), the extract dried down under nitrogen, and the lipid classes separated by thin-layer chromatography (Section 2.3.1.). Radioactivity incorporated into triglycerides during the incubation was then determined as described previously (Section 2.3.1.).

6.2.3. Effect of Methylene Blue on Chronic Alcoholic Fatty Liver:

Male Wistar Albino rats (130-160g initially; University of Surrey strain) were used. For the first four days of the study all animals received the control liquid diet containing no ethanol, that has been described in detail previously (Section 2.2). For the next six days of the study (days 5-11) ethanol was introduced into the diet as 20% of the total calories, and pair-feeding was commenced as described in Section 2.2. For the remainder of the study (days 12-30), ethanol was given as 36% of the total calories in the liquid
diet, control animals being pair-fed diet in which the ethanol calories were replaced by glucose. One group each (n = 12 rats per group) of the ethanol-fed and the pair-fed control animals received liquid diet supplemented with Methylene Blue at the same concentration as that employed by Lieber and DeCarli (1966), namely 130 mg/l diet. Methylene Blue supplementation was commenced on day 5 of the study, and the average daily intake using this method of administration was found to be 42 mg/kg/day. All diets were prepared fresh daily, and dietary intakes recorded daily throughout. Body weights were recorded every two days.

Assessment of the hepatic redox-state: On days 12 and 27 of the study, three animals from each treatment group were killed at midnight so as to assess the redox-state at a time when the animals were maximally intoxicated from ingestion of the ethanol-containing liquid diet. Portions of liver were freeze-clamped in situ within 10-12s of sacrifice and ATP, $\alpha$-glycerophosphate, lactate, pyruvate, 3-hydroxybutyrate, and acetoacetate concentrations measured in the frozen tissue as described previously (Section 2.4). Remaining liver tissue was homogenised in 0.25M sucrose and assayed for triglycerides (Section 2.3). A blood sample was also collected from the necks of the animals for serum ethanol analysis by head-space gas chromatography (Section 2.6).

On day 30 of the experiment, the remaining animals in each group were anaesthetised with diethyl ether, blood collected by closed cardiac puncture and serum prepared. After sacrifice, livers were
excised, rinsed, blotted, weighed and a portion placed in formal-saline for histological assessment using haematoxylin + eosin and Oil Red 'O' staining methods. Remaining liver tissue was homogenised in ice-cold 0.25M sucrose containing 3mM EDTA, and a portion frozen immediately in liquid nitrogen for the determination of reduced glutathione. Total hepatic lipid and triglycerides were determined as described above. Serum was assayed for ethanol by head-space gas chromatography (Section 2.6) and GLDH activity was determined using kits supplied by BCL Ltd (Section 2.10). A modification of the method of Bernt and Bergmeyer (1974) was used to assay reduced glutathione (GSH) in liver homogenates (Section 2.9.3).

6.3 RESULTS

Results of the in vitro liver slice studies are shown in Table 6.1. The presence of ethanol in the incubation medium, at 20 and 40 mM concentrations, decreased the oxidation of [1-^{14}C] palmitate to $^{14}$CO$_2$ by 22% and 43% respectively. Addition of sorbitol to the medium caused an increase in the lactate/pyruvate ratio slightly greater than that caused by either 20 mM or 40 mM ethanol, but did not impair the oxidation of [1-^{14}C] palmitate. On the contrary, fructose and sorbitol enhanced this oxidation. Incorporation of palmitate into triglyceride was increased by sorbitol, although the most marked rise was seen after the addition of 40 mM ethanol to the incubation.
### TABLE 6.1

**EFFECT OF SORBITOL AND ETHANOL ON PALMITATE OXIDATION AND ESTERIFICATION BY RAT LIVER SLICES**

<table>
<thead>
<tr>
<th>Addition</th>
<th>$^{14}$CO$_2$ evolved in incubation (dpm x 10$^{-3}$/g liver)</th>
<th>Radioactivity incorporated into triglycerides (dpm x 10$^{-3}$/g liver)</th>
<th>Lactate/Pyruvate ratio in incubation medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (10 mM)</td>
<td>103.6 ± 5.5 (8)</td>
<td>195.3 ± 5.5 (6)</td>
<td>14.6 ± 0.6 (4)</td>
</tr>
<tr>
<td>Fructose (10 mM)</td>
<td>133.1 ± 2.6 (8)*</td>
<td>190.6 ± 25.0 (6)</td>
<td>13.5 ± 2.5 (4)</td>
</tr>
<tr>
<td>Sorbitol (10 mM)</td>
<td>144.6 ± 7.5 (8)*</td>
<td>227.3 ± 24.9 (6)*</td>
<td>47.4 ± 5.4 (6)*</td>
</tr>
<tr>
<td>Saline</td>
<td>115.8 ± 3.0 (6)</td>
<td>187.0 ± 10.8 (4)</td>
<td>11.7 ± 2.6 (4)</td>
</tr>
<tr>
<td>Ethanol (20 mM)</td>
<td>90.4 ± 6.2 (6)**</td>
<td>288.4 ± 65.6 (4)**</td>
<td>37.9 ± 5.9 (4)**</td>
</tr>
<tr>
<td>Ethanol (40 mM)</td>
<td>65.7 ± 6.3 (6)**</td>
<td>386.7 ± 52.0 (4)**</td>
<td>39.4 ± 0.4 (4)**</td>
</tr>
</tbody>
</table>

Approximately 100 mg of rat liver slices were incubated for 90 mins at 37°C in 3.5 ml Krebs-Henseleit buffer (pH = 7.4) containing 1 μCi albumin-bound [1-14C] palmitic acid. 1.0 ml incubation medium was removed after 60 mins. for determination of lactate and pyruvate concentrations. Results are shown as means ± S.D. with the number of determinations being given in parentheses.

* = p < 0.001 vs. glucose group.  ** = p < 0.001 vs. saline group
TABLE 6.2
EFFECT OF NALOXONE ON THE HEPATIC REDOX-STATE AND TRIGLYCERIDES AFTER ACUTE ETHANOL ADMINISTRATION

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic ATP (nmol/g)</th>
<th>Lactate/Pyruvate</th>
<th>3-Hydroxybutyrate/Acetoacetate</th>
<th>Hepatic Triglyceride (mg/g) 1.5h</th>
<th>Hepatic Triglyceride (mg/g) 3.0h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3726 ± 363</td>
<td>16.5 ± 8.0</td>
<td>2.58 ± 0.30</td>
<td>6.6 ± 1.6</td>
<td>4.9 ± 0.9</td>
</tr>
<tr>
<td>Saline + Naloxone</td>
<td>3423 ± 784</td>
<td>15.7 ± 5.1</td>
<td>2.75 ± 0.49</td>
<td>7.0 ± 2.0</td>
<td>5.5 ± 1.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>3973 ± 758</td>
<td>30.5 ± 8.0*</td>
<td>4.35 ± 0.10*</td>
<td>14.5 ± 4.6*</td>
<td>11.9 ± 4.5+</td>
</tr>
<tr>
<td>Ethanol + Naloxone</td>
<td>4070 ± 785</td>
<td>18.2 ± 3.2**</td>
<td>2.54 ± 0.66**</td>
<td>14.2 ± 2.0*</td>
<td>8.8 ± 2.11**</td>
</tr>
</tbody>
</table>

Determinations were made on liver tissue taken by freeze-clamping at 90 mins. after intraperitoneal injection of ethanol (2 g/kg) or an equivalent volume of saline. Naloxone (2 mg/kg i.p.) was given 1.0 hour, and where appropriate, 2.5 hours, after ethanol. Results are shown as mean ± S.D. n = 6 rats per group. * = p < 0.001 vs. saline only group. ** = p < 0.001 vs. ethanol only group. + = p < 0.01 vs. saline only group. +++ = p < 0.005 vs. saline only group.
In the *in vivo* studies, administration of Naloxone, one hour after ethanol, prevented the disturbances in the hepatic cytosolic and mitochondrial redox-states caused by ethanol, measured as the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios respectively. This correction of the ethanol-induced redox-state changes by Naloxone was not associated with any significant decrease in the hepatic triglyceride accumulation measured either 1.5 hr or 3.0 hr after ethanol (Table 6.2).

Naloxone had an effect on palmitate oxidation when liver slices taken from animals treated with this drug alone were studied *in vitro*. This resulted in an increased esterification of palmitate into triglycerides *in vitro*. While liver slices from ethanol-treated rats showed a lowered oxidation of palmitate, and increased incorporation of palmitate into triglycerides, these phenomena were not reversed by Naloxone pre-treatment (Table 6.3).

Experiments on the hepatic uptake of palmitate *in vivo* showed that ethanol administration caused increased incorporation of this fatty acid into total hepatic lipids and triglycerides. This effect was not influenced by Naloxone treatment, despite the correction of the redox-state changes by the latter compound.

Sorbitol administration produced a significant increase in the hepatic lactate/pyruvate ratio, similar to that observed after ethanol, whilst also causing a 200% increase in the hepatic α-glycerophosphate concentration. Sorbitol also caused an increase in the hepatic 3-hydroxybutyrate/acetoacetate ratio, although this was not
TABLE 6.3

EFFECT OF NALOXONE ON HEPATIC PALMITIC ACID DISPOSAL AFTER ACUTE ETHANOL ADMINISTRATION

<table>
<thead>
<tr>
<th>Treatment</th>
<th>[1-(^{14})C] produced from [1-(^{14})C] palmitate by liver slices in vitro (dpm x 10(^{-3})/g liver)</th>
<th>Radioactivity incorporated into triglyceride in liver slice incubations in vitro (dpm x 10(^{-3})/liver)</th>
<th>% injected [U-(^{14})C] palmitate incorporated into liver lipids in vivo</th>
<th>Radioactivity incorporated into liver triglycerides after in vivo injection of [U-(^{14})C] palmitate (dpm x 10(^{-3})/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>365 ± 56</td>
<td>114 ± 35</td>
<td>8.2 ± 1.0</td>
<td>82.7 ± 10.2</td>
</tr>
<tr>
<td>Saline + Naloxone</td>
<td>233 ± 39*</td>
<td>203 ± 20*</td>
<td>8.2 ± 0.7</td>
<td>90.1 ± 24.7</td>
</tr>
<tr>
<td>Ethanol</td>
<td>216 ± 41**</td>
<td>210 ± 34**</td>
<td>13.9 ± 1.9**</td>
<td>213.5 ± 29.5**</td>
</tr>
<tr>
<td>Ethanol + Naloxone</td>
<td>146 ± 33*</td>
<td>207 ± 63**</td>
<td>13.7 ± 4.5**</td>
<td>234.9 ± 50.5**</td>
</tr>
</tbody>
</table>

90 mins after administration of ethanol (2 g/kg i.p.) animals were either injected with [U-\(^{14}\)C] palmitate (1 \(\mu\)Ci/100 g b.w) and sacrificed ten minutes later for determination of radioactivity incorporated into hepatic lipids, or sacrificed, liver slices prepared, and incubated for 90 mins. in Krebs-Henseleit bicarbonate buffer (pH = 7.4) containing 1 \(\mu\)Ci albumin-bound [1-\(^{14}\)C] palmitic acid. Each value represents the mean ± S.D. of six determinations. * = \(p < 0.01\) vs saline only group. ** = \(p < 0.001\) vs saline only group. + = \(p < 0.001\) vs saline group and N.S. vs ethanol only. ++ = \(p < 0.001\) vs both saline groups and N.S. vs ethanol only. N.S. = not significant.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate/Pyruvate</td>
<td>20.1 ± 2.2</td>
<td>42.0 ± 10.7*</td>
</tr>
<tr>
<td>3-Hydroxybutyrate/Acetoacetate</td>
<td>3.15 ± 0.83</td>
<td>4.97 ± 2.00</td>
</tr>
<tr>
<td>α-Glycerophosphate</td>
<td>98 ± 11</td>
<td>294 ± 22*</td>
</tr>
<tr>
<td>ATP (nmol/g liver)</td>
<td>3986 ± 525</td>
<td>3102 ± 193</td>
</tr>
<tr>
<td>% injected [U-14C] palmitate incorporated into liver lipids</td>
<td>8.0 ± 1.4</td>
<td>8.9 ± 0.9</td>
</tr>
<tr>
<td>[U-14C] Palmitate incorporated into liver triglycerides after in vivo injection (dpm x 10^-2/g liver)</td>
<td>67.3 ± 11.6</td>
<td>103.9 ± 45.5</td>
</tr>
<tr>
<td>[1-14C] palmitate oxidised to 14CO2 in liver slice incubations (dpm x 10^-3/g liver)</td>
<td>17.9 ± 2.3</td>
<td>18.7 ± 5.6</td>
</tr>
<tr>
<td>[1-14C] Palmitate incorporated into triglycerides in liver slice incubations (dpm x 10^-3/liver)</td>
<td>51.9 ± 21.2</td>
<td>123.7 ± 19.2*</td>
</tr>
<tr>
<td>Liver Triglyceride (mg/g)</td>
<td>8.8 ± 1.6</td>
<td>9.7 ± 2.2</td>
</tr>
</tbody>
</table>

90 mins after administration of glucose or sorbitol (3.5 g/kg i.p.), animals were sacrificed, freeze-clamped liver samples taken for metabolite determinations, and liver slices prepared for incubation with albumin-bound [1-14C] palmitic acid. In experiments where in vivo incorporation of [U-14C] palmitate was measured, the label was injected (1 μCi/100 g b.w.) 90 mins after glucose or sorbitol dosing and animals sacrificed 10 minutes later. Values are means ± S.D. n = 5 rats per group. * = p < 0.001 vs. glucose group.
statistically significant due to the wide individual variation in the extent of this effect. These changes in the hepatic redox-state observed after sorbitol were not associated with an increased incorporation of \([U-^{14}C]\) palmitate into liver lipids or triglycerides, or increased hepatic triglyceride concentrations. The oxidation of palmitate by liver slices from rats dosed with sorbitol was not different from that obtained with control animals which received glucose. Sorbitol did, however, cause increased esterification of palmitate into triglycerides in these incubations (Table 6.4).

Various biochemical components associated with the hepatic redox-state were determined during the chronic Methylene Blue experiment, and the results are shown in Table 6.5. At the beginning of administration of 36% of the total calories as ethanol (day 12), ethanol-fed animals showed 154% and 249% increases in the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios respectively compared with their pair-fed controls. Ethanol caused a 65% increase in the hepatic \(\alpha\)-glycerophosphate concentration, but did not affect hepatic ATP concentrations at this time point. Methylene Blue alone did not affect any of these parameters in pair-fed controls at this stage of the experiment, but significantly reversed some of the redox-state changes observed in ethanol-fed animals. Partial correction of the lactate/pyruvate ratio was achieved by Methylene Blue, whilst the 3-hydroxybutyrate/acetoacetate ratio, and \(\alpha\)-glycerophosphate concentrations were returned virtually to control values.
After 16 days of ethanol feeding (i.e., day 27 of the study), the redox-state changes observed in the ethanol-fed rats were less marked than at day 12, the increases in the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios being 59% and 38% when ethanol-fed rats were compared with their pair-fed controls. The hepatic α-glycerophosphate concentration was increased by 37% in ethanol-treated rats. The ethanol-induced increase in the 3-hydroxybutyrate/acetoacetate ratio was not statistically significant at this time point, possibly as a result of the small number of animals studied. Once again, Methylene Blue corrected these redox-state changes in ethanol-fed animals, although the effect on the lactate/pyruvate ratio was not statistically significant. Ethanol alone did not affect ATP concentrations at day 27, whereas Methylene Blue caused a slight decrease in this parameter in both controls and ethanol-fed animals. Serum ethanol concentrations were unaffected by Methylene Blue on both day 12 and day 27 of the study.

Hepatic concentrations of individual redox-pair metabolites during the study are shown in Table 6.6. Methylene Blue alone increased lactate and acetoacetate concentrations on day 12 of the study, although no effect was observed on day 27. Hepatic lactate and pyruvate concentrations were consistently lower in the ethanol-fed animals. Methylene Blue corrected the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios in ethanol-fed animals on day 12 through increases in hepatic pyruvate and acetoacetate concentrations, and by decreasing the 3-hydroxybutyrate concentration. Ethanol
feeding caused significant increases in hepatic 3-hydroxybutyrate concentrations at both time points studied.

Hepatic triglyceride content at these time points in the study are shown in Table 6.7. On day 12 there was no significant difference in triglyceride contents between any of the groups, whereas on day 27, there was a significant increase in the ethanol-fed rats, irrespective of whether they had received Methylene Blue supplements.

Hepatic lipid, body weight, ethanol intake and other determinations made at the end of the study are shown in Table 6.8. There was no difference in body weight gain between any of the groups and Methylene Blue did not affect ethanol intake. Ethanol feeding caused slight enlargement of the livers, which was not prevented by Methylene Blue supplementation. Ethanol feeding caused 89% and 196% increases respectively in the total hepatic lipid and hepatic triglyceride concentrations, which were not significantly influenced by Methylene Blue. Ethanol caused a slight decrease in the reduced glutathione content of the liver but, again, this was not affected by Methylene Blue. Serum ethanol concentrations and the elevated serum glutamate dehydrogenase activities observed in ethanol-fed animals were also not affected by Methylene Blue. Histological examination of liver tissue revealed the presence of the characteristic centrilobular fatty infiltration of alcoholic steatosis in all the ethanol-fed animals, the extent or intralobular distribution of the accumulated fat not being affected by Methylene Blue supplementation.
### TABLE 6.5 EFFECT OF METHYLENE BLUE SUPPLEMENTATION OF ETHANOL-CONTAINING LIQUID DIETS ON THE HEPATOCYTOPLURAL REDOX-STATE DURING CHRONIC ETHANOL FEEDING

<table>
<thead>
<tr>
<th>Day of Study</th>
<th>Measurement</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(A) Control</td>
</tr>
<tr>
<td>12</td>
<td>ATP (nmol/g)</td>
<td>2991 ± 187</td>
</tr>
<tr>
<td></td>
<td>&gt; -glycerophosphate</td>
<td>189 ± 21</td>
</tr>
<tr>
<td></td>
<td>Lactate/pyruvate</td>
<td>12.4 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxybutyrate/acetoacetate</td>
<td>2.34 ± 1.10</td>
</tr>
<tr>
<td></td>
<td>Serum ethanol (mg/100 ml)</td>
<td>-</td>
</tr>
<tr>
<td>27</td>
<td>ATP (nmol/g)</td>
<td>3405 ± 211</td>
</tr>
<tr>
<td></td>
<td>&gt; -glycerophosphate</td>
<td>212 ± 28</td>
</tr>
<tr>
<td></td>
<td>Lactate/pyruvate</td>
<td>16.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxybutyrate/acetoacetate</td>
<td>2.39 ± 0.70</td>
</tr>
<tr>
<td></td>
<td>Serum ethanol (mg/100 ml)</td>
<td>-</td>
</tr>
</tbody>
</table>

Rats were fed with ethanol as part of a liquid diet in which 36% of the total energy was derived from ethanol. Pair-fed controls received diet in which glucose replaced the ethanol energy. Animals were killed at midnight on either day 12 or day 27 of the study, and freeze-clamped liver samples were analysed for metabolites as described in the Methods section. Results are shown as mean ± S.D. (n = 3 rats per group). * = p < 0.001 vs. (A) ** = p < 0.01 vs. (A) + = p < 0.001 vs. (C) ++ = p < 0.01 vs. (C). Abbreviation: MB, Methylene Blue.
## Table 6.6. Effect of Methylene Blue Supplementation of Ethanol-Containing Liquid Diets on the Hepatic Concentrations of Redox Pair Metabolites

<table>
<thead>
<tr>
<th>Day of Study</th>
<th>Measurement</th>
<th>(A) Control</th>
<th>(B) Control + MB</th>
<th>(C) Ethanol</th>
<th>(D) Ethanol + MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>1544 ± 301</td>
<td>2859 ± 504*</td>
<td>736 ± 102*</td>
<td>1009 ± 139</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>125 ± 21</td>
<td>167 ± 31</td>
<td>23 ± 9*</td>
<td>46 ± 13*</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxybutyrate</td>
<td>119 ± 57</td>
<td>144 ± 37</td>
<td>385 ± 93*</td>
<td>242 ± 40*</td>
</tr>
<tr>
<td></td>
<td>Acetoacetate</td>
<td>51 ± 20</td>
<td>98 ± 19*</td>
<td>47 ± 14</td>
<td>80 ± 17*</td>
</tr>
<tr>
<td>27</td>
<td>Lactate</td>
<td>2639 ± 325</td>
<td>2014 ± 393</td>
<td>662 ± 98*</td>
<td>828 ± 107</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>157 ± 14</td>
<td>131 ± 29</td>
<td>25 ± 11*</td>
<td>39 ± 12</td>
</tr>
<tr>
<td></td>
<td>3-Hydroxybutyrate</td>
<td>123 ± 20</td>
<td>159 ± 23</td>
<td>207 ± 31*</td>
<td>176 ± 29</td>
</tr>
<tr>
<td></td>
<td>Acetoacetate</td>
<td>51 ± 14</td>
<td>89 ± 17</td>
<td>63 ± 12</td>
<td>83 ± 17</td>
</tr>
</tbody>
</table>

Experimental details and expression of results are as given in the legend to Table 6.5. Abbreviation: MB, Methylene Blue. All values are in nmol/g wet wt. tissue. (n = 3 rats per group) * = p < 0.001 vs. (A), + = p < 0.001 vs. (C).
TABLE 6.7. EFFECT OF METHYLENE BLUE SUPPLEMENTATION OF ETHANOL-CONTAINING LIQUID DIETS ON HEPATIC TRIGLYCERIDE CONTENT DURING CHRONIC ETHANOL FEEDING

<table>
<thead>
<tr>
<th>Group</th>
<th>(A) Control</th>
<th>(B) Control + MB</th>
<th>(C) Ethanol</th>
<th>(D) Ethanol + MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 12</td>
<td>11.0 ± 3.4</td>
<td>13.0 ± 2.0</td>
<td>15.5 ± 5.3</td>
<td>20.9 ± 8.5</td>
</tr>
<tr>
<td>Day 27</td>
<td>13.5 ± 4.0</td>
<td>16.8 ± 4.0</td>
<td>50.3 ± 11.1*</td>
<td>46.9 ± 8.7*</td>
</tr>
</tbody>
</table>

Determinations were made on liver tissue taken at the same time as freeze-clamped samples for redox-state measurements were prepared as described in legend to Table 6.5. Results are shown as mean ± S.D. (n = 3 rats per group). Abbreviation: MB, Methylene Blue. * = $p < 0.001$ vs. (A) and versus day 12 value for same group.
TABLE 6.8. EFFECT OF METHYLENE BLUE SUPPLEMENTATION OF ETHANOL-CONTAINING LIQUID DIETS ON THE HEPATIC LIPID CONTENT AFTER CHRONIC ETHANOL FEEDING

<table>
<thead>
<tr>
<th>Measurement</th>
<th>(A) Control</th>
<th>(B) Control + MB</th>
<th>(C) Ethanol</th>
<th>(D) Ethanol + MB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in body wt. during study (g)</td>
<td>96.4 ± 8.8</td>
<td>90.0 ± 3.0</td>
<td>80.0 ± 22.0</td>
<td>83.6 ± 9.5</td>
</tr>
<tr>
<td>Ethanol intake (g/day/kg)</td>
<td>-</td>
<td>-</td>
<td>15.1 ± 3.9</td>
<td>14.8 ± 2.4</td>
</tr>
<tr>
<td>Liver wt. (g/100 g body wt)</td>
<td>3.29 ± 0.11</td>
<td>3.64 ± 0.44</td>
<td>3.64 ± 0.35*</td>
<td>3.73 ± 0.14**</td>
</tr>
</tbody>
</table>

Liver

| Total lipids (mg/g)                   | 48.0 ± 10.7 | 52.5 ± 8.4       | 90.7 ± 10.8** | 79.1 ± 11.9**   |
| Triglyceride (mg/g)                   | 15.6 ± 8.6  | 19.8 ± 6.2       | 46.1 ± 13.3** | 38.5 ± 6.7**    |
| Reduced glutathione (µmol/g)          | 6.11 ± 0.44 | 5.46 ± 0.28      | 4.21 ± 0.81*  | 4.58 ± 1.10     |
| Serum ethanol (mg/100 ml)             | -           | -                | 160 ± 83      | 158 ± 61        |
| Serum GLDH (U/l)                      | 2.7 ± 1.5   | 1.4 ± 1.1        | 9.4 ± 5.8*    | 9.3 ± 5.5*      |

Rats were given ethanol as part of a liquid diet in which 36% of the total energy was derived from ethanol. Pair-fed controls received diet in which glucose replaced the ethanol energy. Methylene Blue was added to the diets at a concentration of 130 mg/l, to give an average daily intake of 42 mg per day/kg. Animals were killed after 20 days of receiving 36% of their energy as ethanol (ie: day 30 of the study). Results are shown as mean ± S.D. (n = 6 rats per group). Abbreviation: MB, Methylene Blue. * = p < 0.025 vs. (A). ** = p < 0.001 vs. (A). † = p < 0.005 vs. (A).
6.4 DISCUSSION

The results of these experiments cast some doubts on the role of the altered hepatic redox-state as a mechanism of the acute and chronic ethanol-induced hepatic triglyceride accumulation. Data obtained from the studies in vitro (Table 6.1) suggest that there is no apparent association between the increased lactate/pyruvate ratio, and the decrease in palmitate oxidation in the presence of ethanol. Under these conditions, in vitro elevation of the cytosolic NADH:NAD ratio by either ethanol or sorbitol occurred in parallel with increased incorporation of palmitate into triglycerides. However, the fact that the higher concentration of ethanol (40 mM) was much more effective at increasing palmitate esterification, without further raising the lactate/pyruvate ratio above that found in the presence of 20 mM ethanol, suggested that factors other than the redox-state alteration might be implicated. Experiments in vivo, with Naloxone, also support these conclusions drawn from the in vitro findings. Correction of the redox-state in ethanol-treated rats by Naloxone did not prevent the hepatic triglyceride accumulation, measured 1.5 hr after acute ethanol administration (Table 6.2). This may have been the consequence of administration of the drug 1.0 hr after ethanol, so that the redox-state disturbance in ethanol-treated animals prior to Naloxone administration could have contributed to the triglyceride deposition. Prolonging the period of study to 3.0 hr after ethanol, however, with a further Naloxone dose given at 2.5 hr, since the drug
has a short half-life in the rat (Weinstein et al. 1974), still revealed that the drug did not prevent completely the increase in hepatic triglycerides caused by ethanol, although a non-significant, downward trend was observed.

The experiments on the in vivo incorporation of radio-labelled palmitate into hepatic lipids also indicate that the redox-state change following ethanol was not responsible for the observed increase in triglyceride synthesis (Tables 6.3 and 6.4). This probably cannot be attributed to any difference between the ethanol-treated and control animals in the specific activity of $^{14}$C-palmitate, since a dose of ethanol, identical to that used here, has been shown to have no influence on the hepatic free fatty acid pool, serum free fatty acid concentration, or the flux of fatty acids from the peripheral tissues (Abrams and Cooper, 1976a; Abrams and Cooper, 1976b). Naloxone treatment failed to reverse these ethanol-mediated changes in the in vivo incorporation of palmitate into liver triglycerides, whilst sorbitol only caused a small, non-significant increase. Sorbitol also increased the $\alpha$-glycerophosphate concentration in vivo, as was expected from the observed effects of this substance on the redox-state. However, this did not have much bearing on triglyceride formation in vivo (Table 6.4).

The data obtained from experiments with liver slices taken from animals dosed with either ethanol or sorbitol, suggest that the redox-state does not influence the hepatic fatty acid oxidising capacity. Sorbitol administration did not affect the ability of
liver tissue to oxidise palmitate to \( \text{CO}_2 \), when values were compared with those found with control animals which were given glucose. Interpretation of the results from Naloxone-dosed rats, on the other hand, presented a problem, since the drug alone tended to impair palmitate oxidation. This effect of the drug was also associated with increased esterification of this fatty acid into triglyceride by the liver slices. This action of Naloxone did not appear, however, to influence incorporation of injected palmitate into liver lipids \textit{in vivo} (Table 4). This observation raises the question as to whether the decreased fatty acid oxidation rate after ethanol reported here and previously (Lieber and Schmid, 1961; Ontko, 1973; Blomstrand \textit{et al}, 1973) has any role in the hepatic triglyceride accumulation. However, in a recent study it was reported that administration of 4-pentenoic acid, a specific inhibitor of \( \beta \)-oxidation, to rats increased hepatic triglyceride concentrations, the increases being quantitatively related to the degree of inhibition of fatty acid oxidation (Thayer, 1984).

The mechanism by which Naloxone corrects the ethanol-induced hepatic redox-state changes is not clear from this study, but in view of the lack of effect of the drug on ATP concentrations, non-enzymatic oxidation of NADH or simple uncoupling of oxidative phosphorylation do not seem to be involved. The apparent inhibition of \( \beta \)-oxidation by the drug could be a contributory factor, since this may cause sparing of hepatic NAD. However, no effect of the drug alone on the redox-state was observed in control animals. It is clear from the
blood ethanol data reported later (Chapter 8), that inhibition of ethanol metabolism is not concerned with the action of Naloxone on the redox-state.

With regard to the decrease in palmitate oxidation by liver slices from ethanol-treated animals, the underlying mechanism may involve mitochondrial injury, rather than direct acute effects of ethanol, including changes in the redox-state, being implicated. Such mitochondrial damage, as discussed earlier, could arise from covalent binding of ethanol-derived acetaldehyde with mitochondrial protein (Tuma and Sorrell, 1985), or peroxidative injury to the organelle (Rouach et al, 1983).

Some evidence for the latter mechanism being of importance comes from the work described in the next chapter, in which antioxidants have been found to counteract many of the altered patterns of fatty acid disposal and increased hepatic triglyceride levels after ethanol dosing. Furthermore, antioxidants achieved this without reversing the ethanol-induced hepatic redox-state changes, adding more weight to the argument that ethanol-induced hepatic triglyceride accumulation may not be related to the hepatocellular NADH:NAD ratio.

One possible consequence of the redox-state change that might contribute to the triglyceride deposition is increased fatty acid synthesis resulting from increased NADPH levels (Lieber and Schmid, 1961). Although this has not been directly studied here, one recent study in which rates of lipogenesis were measured using tritiated water, showed that liver slices from ethanol-fed animals in fact
showed lower rates of lipogenesis than those from control, pair-fed rats (Venkatesan et al, 1985).

The conclusions from these acute studies are similar to those to be drawn from the chronic Methylene Blue study. The results of the latter show that supplementation of ethanol-containing liquid diets with Methylene Blue can largely prevent the hepatocellular redox-state changes induced by chronic ethanol feeding in the rat. These redox-state changes are however, less marked than those that are often observed after acute ethanol administration to naive rats, and they become attenuated after long-term feeding, confirming previous reports (Domschke et al, 1974; Saluspuro et al, 1981). In this study, this attenuation of ethanol-induced redox-state changes between day 12 and day 27 occurred in spite of an increase in ethanol consumption by the animals in the intervening period, and the fact that serum ethanol concentrations were slightly higher at the later time point.

The mechanism by which Methylene Blue corrects the ethanol-induced redox-state changes is thought to be through non-enzymatic oxidation of NADH. This may, in turn, affect availability of NADH for mitochondrial NADH oxidation and oxidative phosphorylation. This is indicated to some extent by the slightly lower ATP concentrations found on day 27 in the Methylene Blue treated animals. This decrease was not sufficient, however, to cause any evidence of deranged hepatocyte function, for example in the form of increased liver lipid contents or serum glutamate dehydrogenase activities, in control animals. Another possible consequence of the
action of Methylene Blue might be decreased reduced glutathione concentrations as a result of lower NADPH concentrations to act as a cofactor for glutathione reductase. This, in turn, could possibly affect the susceptibility of hepatocytes to injury from toxins such as ethanol. Acute injections of Methylene Blue can cause hepatic glutathione depletion in mice (Hrushesky et al, 1985). However, in the present study there was no evidence of Methylene Blue supplementation of the diet affecting hepatic reduced glutathione concentrations in either controls or ethanol-fed animals.

The concentrations of the individual redox-pair metabolites (Table 6.6) show that the lower carbohydrate content of the ethanol-containing diet causes decreases in the hepatic lactate and pyruvate concentrations when compared to the values obtained in pair-fed controls. Under these circumstances, there may be more dependence on fatty acid oxidation for hepatic energy production, hence ketone body concentrations may increase simultaneously, providing that β-oxidation of fatty acids to two-carbon fragments is not inhibited by ethanol. In this study, 3-hydroxybutyrate concentrations were higher in ethanol-fed animals, but probably only as a consequence of the increased mitochondrial NADH:NAD ratio. Overall, taking this into account, the ketone body concentrations were comparable between the control and ethanol-fed rats, indicating that β-oxidation itself may not be affected by ethanol feeding. However, the complete oxidation of fatty acids to CO₂ may be impaired at the tricarboxylic acid cycle stage, and thus be an important factor.
in the production of steatosis, as previously proposed by Lieber and Schmid (1961). This may arise after chronic ethanol intake through irreversible injury to the mitochondria (Cederbaum and Rubin, 1975), this being indicated in the present study by the elevated serum glutamate dehydrogenase activities in ethanol-fed animals, this enzyme being localized in the mitochondrial matrix.

The fact that Methylene Blue only caused partial correction of the cytosolic NADH:NAD ratio in ethanol-fed animals might indicate that the decrease in this ratio was a contributory factor in the production of steatosis. However, probably the most important consequence of the decrease in this ratio, that in turn might influence triglyceride synthesis, is the increase in α-glycerophosphate concentrations during ethanol feeding. In this respect, concentrations of this metabolite were restored almost to control values by Methylene Blue without hepatic lipid content being decreased. The investigation of hepatic lipid concentrations clearly show that despite amelioration of the ethanol-induced redox-state changes, Methylene Blue does not prevent the fatty infiltration of the liver after chronic ethanol intake.

This experiment therefore suggests, as for the acute studies, that the increased NADH:NAD ratio resulting from ethanol oxidation is not the primary mechanism by which fatty liver is induced after long-term intake, and that the metabolic disturbances resulting from the redox-state change, including increased α-glycerophosphate concentrations, may not be implicated in ethanol-induced steatosis.
This view is supported by the finding in the present study that there was no significant increase in hepatic triglycerides on the day when the ethanol-induced redox-state changes were most marked (ie: day 12), whereas triglyceride accumulation was observed on day 27 when the ethanol-induced redox-state changes were attenuated.

In conclusion, these results confirm what was suspected from the acute experiments, namely that (+)-catechin and 3-palmitoyl-(+)-catechin do not protect against ethanol hepatotoxicity, either acute or chronic, through their ability to correct the ethanol-induced redox-state changes. It now seems likely that their mechanism of action involves counteraction of some other ethanol-induced event, the most probable explanation in view of the cumulative findings, being inhibition of lipid peroxidation due to these drug's free radical-scavenging properties. In order to test this hypothesis, in the next chapter the influence of standard synthetic antioxidants on the redox-state, and hepatic lipid disposal after acute ethanol dosing, and the effect of antioxidant administration on the chronic ethanol-induced fatty liver has been studied, using identical methods to those employed here.
Chapter 7

THE INFLUENCE OF ANTIOXIDANTS ON THE HEPATIC CHANGES AFTER ACUTE AND CHRONIC ETHANOL ADMINISTRATION

7.1 INTRODUCTION:

The work in this chapter aims to assess the possible role of the antioxidant properties of the (±)-catechin compounds, as opposed to their redox-state modulating properties, in preventing acute and chronic ethanol-induced fat accumulation. This has been investigated by examining the effects of synthetic antioxidants on the parameters measured after acute ethanol administration in the experiments with the (±)-catechin compounds (Chapters 3 and 5), namely, the redox-state, triglyceride levels, and palmitate oxidation by liver slices taken after ethanol dosing. Hepatic uptake and incorporation of [U-14C] palmitic acid was not studied in these experiments, due to some uncertainty about the effect of injecting antioxidants as emulsions intraperitoneally on the hepatic uptake of fatty acids injected by the same route, as this has been shown to be an interfering factor in some studies (Abrams and Cooper, 1976b).

In addition to these experiments on the effect of antioxidants on the redox-state changes and lipid metabolism, the possible protective effect of these substances against the acute and chronic forms of
fatty liver, has been assessed. This will allow direct comparison with the effects of the (+)-catechin compounds in these experimental situations, as described in Chapter 3. In the acute studies, the two antioxidants employed were Butylated Hydroxytoluene (BHT) and N,N'-Diphenyl-p-phenylenediamine (DPPD).

In the chronic fatty liver experiment, only DPPD was investigated, since long-term administration of BHT is known to be associated with hepatomegaly, enzyme induction and other effects which may complicate interpretation of the results in ethanol-fed animals (Babich, 1982).

7.2 METHODS

(i) Acute studies: BHT and DPPD were prepared as emulsions in 0.5% (w/v) acacia gum containing 0.1% (w/v) Tween 80, and given at doses of 400 mg/kg (i.p.) at 24 and 1 hours before ethanol administration (2 g/kg i.p.). Fasted rats were used as described previously, and appropriate controls were sham-dosed with vehicle substances at the same time-points at which ethanol and/or antioxidants were administered. Animals were sacrificed 90 mins. after ethanol, a portion of liver freeze-clamped for metabolite determinations, liver slices prepared for incubation with [1-14C] palmitic acid, and the remaining liver tissue homogenised for determination of triglyceride concentrations. Some animals were sacrificed 3 hours after ethanol dosing for triglyceride measurements, so as to assess the effect of
antioxidants on ethanol-induced lipid accumulation over a slightly longer time period. All analytical methods and measurement of $^{14}\text{CO}_2$ production from [1-$^{14}\text{C}$] palmitate were performed as described previously (Chapter 5).

The effects of BHT and DPPD on the fatty liver produced by a large oral dose of ethanol were also investigated in fasted rats. DPPD or BHT were given (400 mg/kg i.p.), as described above, at 24 and 1 hours before ethanol (6 g/kg orally as a 50% (v/v) solution). Glucose-dosed controls were employed, and animals were sacrificed 16 hours after ethanol dosing for the measurement of liver triglycerides (see Chapter 3).

(iii) Chronic studies: The effect of DPPD on chronic alcoholic fatty liver production was studied using the 'Complan' liquid diet regime already described in detail (section 2.2.). Groups of eight male Wistar albino rats (University of Surrey strain) were given ethanol as 36% of the total calories for a period of 21 days following an initial introductory period to the liquid diet, appropriate controls being pair-fed diet in which the ethanol calories were replaced by glucose. DPPD was given once weekly (400 mg/kg i.p.) in fresh corn oil, this dosing regime being sufficient to maintain effective levels of the substance in the body between doses, since the compound forms depots in the body fat, and does not undergo any significant hepatic metabolism. Dietary intakes and body weights were recorded throughout. On the last day of the study, animals were bled by closed cardiac puncture under ether anaesthesia, serum prepared, and
the animals sacrificed. Livers were removed, weighed and homogenised in 0.25M sucrose. Serum ethanol, glutamate dehydrogenase activity (GLDH), total hepatic lipid, and liver triglycerides were determined as described previously. Microsomes were prepared from the livers of the animals as described in section 2.8. Susceptibility to in vitro lipid peroxidation was determined by incubating microsomes in the presence of the hydroxyl radical generating system (NADPH/Fe$^{2+}$/ADP), exactly as described for the testing of antioxidant activity of various scavengers, as described in Chapter 4 and section 2.8. Microsomal lipid peroxidation was expressed as absorbance at 535 nm/mg microsomal protein, the production of thiobarbituric acid reactive material being determined after a 15 minute incubation of microsomes with NADPH/Fe$^{2+}$/ADP. Microsomal protein was determined by the method of Lowry et al (1951) using bovine serum albumin as the reference.

7.3 RESULTS

(i) Acute studies: The effect of BHT on the parameters studied after acute ethanol administration is shown in Table 7.1. Neither ethanol or BHT, alone or in combination, had any effect on liver ATP concentrations. As found previously, ethanol caused an increase in the lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios, these changes being unaffected by BHT administration. However, ethanol caused 96% and 128% increases in the hepatic triglyceride concentrations measured at 90 and 180 mins. after dosing respectively,
<table>
<thead>
<tr>
<th>Determination</th>
<th>A. Control</th>
<th>B. Control + BHT</th>
<th>C. Ethanol</th>
<th>D. Ethanol + BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver ATP (nmol/g)</td>
<td>3584 ± 413</td>
<td>3592 ± 272</td>
<td>3726 ± 279</td>
<td>3860 ± 584</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>14.4 ± 1.2</td>
<td>16.5 ± 2.7</td>
<td>40.7 ± 9.8*</td>
<td>50.2 ± 11.3</td>
</tr>
<tr>
<td>3-Hydroxybutyrate/Acetoacetate</td>
<td>2.12 ± 0.24</td>
<td>2.12 ± 0.28</td>
<td>5.05 ± 0.32*</td>
<td>6.07 ± 0.95</td>
</tr>
<tr>
<td>Triglyceride (90 mins) (mg/g)</td>
<td>5.7 ± 1.7</td>
<td>5.6 ± 0.5</td>
<td>11.2 ± 1.5*</td>
<td>5.4 ± 0.8**</td>
</tr>
<tr>
<td>Triglyceride (180 mins) (mg/g)</td>
<td>4.9 ± 0.9</td>
<td>3.8 ± 1.1</td>
<td>11.9 ± 3.4*</td>
<td>4.1 ± 1.8**</td>
</tr>
<tr>
<td>14CO₂ production from [1-14C] palmitate by liver slices (dpm x 10⁻²/g liver)</td>
<td>350 ± 43</td>
<td>411 ± 56</td>
<td>212 ± 28*</td>
<td>300 ± 53+</td>
</tr>
</tbody>
</table>

All determinations, except where indicated, were carried out 90 mins. after ethanol administration (2 g/kg i.p.). BHT was given (400 mg/kg i.p.) at 24 and 1 hours before ethanol. Results are shown as mean ± S.D. n = 5 animals per group. * = p < 0.001 vs. A. ** = p < 0.001 vs. C. + = p < 0.005 vs. C.
TABLE 7.2  THE EFFECT OF N,N',-DIPHENYL-P-PHENYLENEDIAMINE (DPPD) ON THE HEPATIC REDOX-STATE, TRIGLYCERIDE CONTENT AND PALMITATE OXIDATION AFTER ACUTE ETHANOL ADMINISTRATION

<table>
<thead>
<tr>
<th>Determination</th>
<th>A. Control</th>
<th>B. Control + DPPD</th>
<th>C. Ethanol</th>
<th>D. Ethanol + DPPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP (nmol/g)</td>
<td>3546 ± 385</td>
<td>3536 ± 270</td>
<td>3733 ± 214</td>
<td>3757 ± 38</td>
</tr>
<tr>
<td>Lactate/Pyruvate</td>
<td>14.4 ± 1.2</td>
<td>16.7 ± 3.4</td>
<td>41.9 ± 7.2*</td>
<td>48.7 ± 13.0</td>
</tr>
<tr>
<td>3-Hydroxybutyrate/Acetoacetate</td>
<td>2.12 ± 0.24</td>
<td>2.33 ± 0.47</td>
<td>5.00 ± 0.24*</td>
<td>5.08 ± 0.47</td>
</tr>
<tr>
<td>Triglyceride (90 mins) (mg/g)</td>
<td>5.7 ± 1.7</td>
<td>6.5 ± 1.8</td>
<td>10.7 ± 1.4*</td>
<td>6.9 ± 0.6**</td>
</tr>
<tr>
<td>Triglyceride (100 mins) (mg/g)</td>
<td>4.9 ± 0.9</td>
<td>5.2 ± 3.4</td>
<td>11.9 ± 3.4*</td>
<td>3.5 ± 0.9**</td>
</tr>
<tr>
<td>$^{14}$CO$_2$ production from [1-$^{14}$C] palmitate by liver slices</td>
<td>332 ± 52</td>
<td>369 ± 29</td>
<td>194 ± 35*</td>
<td>293 ± 44+</td>
</tr>
</tbody>
</table>

All determinations, except where indicated, were carried out on tissue taken 90 mins. after ethanol administration (2 g/kg i.p.). DPPD was given (400 mg/kg i.p.) at 24 and 1 hours before ethanol. Results are shown as mean ± S.D. n = 5 animals per group. * = p < 0.001 vs. A. ** = p < 0.001 vs. C. + = p < 0.02 vs. C.
this accumulation being completely prevented by BHT. BHT alone had no effect on hepatic triglyceride concentrations. Ethanol caused a 40% decrease in the ability of liver slices taken at sacrifice to oxidise [1-\(^{14}\)C] palmitate to \(^{14}\)CO\(_2\), the extent of this inhibition being reduced to 15% when BHT was given with ethanol.

An almost identical pattern of results was seen when DPPD was given to ethanol-dosed rats (Table 7.2). This antioxidant also failed to reverse the ethanol-induced increases in the hepatic lactate/pyruvate and 3-hydroxybutyrate/acetoacetate ratios, yet prevented the hepatic triglyceride accumulation, and partially restored the ability of liver tissue to oxidise palmitate to CO\(_2\), after ethanol administration.

The results from the acute alcoholic fatty liver study are shown in Table 7.3. Ethanol administration (6 g/kg p.o.) caused a 128% increase in the hepatic triglyceride content measured 16 hours after dosing, the extent of this accumulation being reduced to 35% and 38% if ethanol-dosed animals were pre-treated with BHT or DPPD respectively.

(ii) Chronic study: The results from the chronic ethanol feeding study are shown in Table 7.4. DPPD dosing did not affect body weight gain, ethanol intake, liver weights or serum ethanol concentrations at sacrifice in ethanol-treated rats. Ethanol feeding caused 100% and 250% increases in the total hepatic lipid and triglycerides respectively, DPPD administration reducing the extent of these increases to 25% and 76% in ethanol-fed animals. There were some
TABLE 7.3 EFFECTS OF BHT AND DPPD ON THE HEPATIC TRIGLYCERIDE ACCUMULATION FOLLOWING A LARGE ACUTE ORAL ETHANOL DOSE

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hepatic Triglyceride 16h after Ethanol (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>10.4 ± 2.1</td>
</tr>
<tr>
<td>Glucose + BHT</td>
<td>12.1 ± 2.0</td>
</tr>
<tr>
<td>Glucose + DPPD</td>
<td>9.8 ± 1.3</td>
</tr>
<tr>
<td>Ethanol only</td>
<td>23.7 ± 0.4*</td>
</tr>
<tr>
<td>Ethanol + BHT</td>
<td>14.1 ± 3.8**</td>
</tr>
<tr>
<td>Ethanol + DPPD</td>
<td>14.4 ± 3.7**</td>
</tr>
</tbody>
</table>

BHT and DPPD were given (400 mg/kg i.p.) at 24 and 1 hours before ethanol (6 g/kg orally). Results are shown as mean ± S.D. (n = 6 rats per treatment group). * = p < 0.001 vs. glucose group. ** = p < 0.001 vs. ethanol only group.
TABLE 7.4  EFFECT OF N,N'-DIPHENYL-P-PHENYLENEDIAMINE (DPPD) ON THE FATTY LIVER PRODUCED BY CHRONIC ETHANOL FEEDING IN THE RAT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>A. Pair-fed Controls</th>
<th>B. Pair-fed Controls + DPPD</th>
<th>C. Ethanol Only</th>
<th>D. Ethanol + DPPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain during study (g)</td>
<td>83 ± 14</td>
<td>84 ± 16</td>
<td>79 ± 18</td>
<td>77 ± 20</td>
</tr>
<tr>
<td>Mean ethanol intake (g/kg/day)</td>
<td>-</td>
<td>-</td>
<td>12.5 ± 1.1</td>
<td>12.6 ± 0.9</td>
</tr>
<tr>
<td>Liver weight (% b.w.)</td>
<td>3.23 ± 0.15</td>
<td>3.45 ± 0.25</td>
<td>3.46 ± 0.11</td>
<td>3.45 ± 0.17</td>
</tr>
<tr>
<td>Serum ethanol at sacrifice (mg/100 ml)</td>
<td>-</td>
<td>-</td>
<td>182 ± 53</td>
<td>170 ± 78</td>
</tr>
<tr>
<td>Liver lipids (mg/g)</td>
<td>44.9 ± 2.7</td>
<td>46.7 ± 2.5</td>
<td>90.0 ± 14.6*</td>
<td>56.0 ± 6.5*</td>
</tr>
<tr>
<td>Liver triglycerides (mg/g)</td>
<td>11.9 ± 1.3</td>
<td>12.1 ± 2.2</td>
<td>41.9 ± 10.2*</td>
<td>21.0 ± 5.4**</td>
</tr>
<tr>
<td>Serum GLDH (U/l)</td>
<td>2.2 ± 1.1</td>
<td>5.0 ± 3.2</td>
<td>10.0 ± 6.3</td>
<td>6.4 ± 3.6</td>
</tr>
<tr>
<td>Microsomal lipid peroxidation (A535/mg microsomal protein)</td>
<td>0.243 ± 0.030</td>
<td>0.047 ± 0.021*</td>
<td>0.286 ± 0.042</td>
<td>0.039 ± 0.019*</td>
</tr>
</tbody>
</table>

DPPD was given once weekly (400 mg/kg i.p.) during the feeding of ethanol as 36% of the total calories in a liquid diet over a period of 21 days. Controls were pair-fed diet in which glucose replaced the ethanol calories. Results are shown as mean ± S.D. (n = 8 rats per group). * = p < 0.001 vs. A. * = p < 0.001 vs. C. ** = p < 0.005 vs. C.
trends evident in the serum GLDH data, ethanol feeding causing an increase, and DPPD slightly counteracting this in ethanol-fed rats, although none of these changes were statistically significant due to the wide individual variation in the values obtained. Ethanol feeding caused only a small, non-significant increase in the susceptibility of liver microsomes to peroxidation by NADPH/ADP/Fe^{2+} in vitro, but microsomes isolated from DPPD-treated animals, both pair-fed controls and ethanol-fed rats, were substantially protected against in vitro peroxidation.

7.4 DISCUSSION

The experiments described here have shown that standard antioxidant substances can prevent the hepatic triglyceride accumulation following acute doses of ethanol, and partly prevent the depressed fatty acid oxidation capability of liver tissue after ethanol dosing, without reversing the increase in the cytosolic or mitochondrial NADH:NAD ratio caused by ethanol metabolism. Subsequent experiments, described later (Chapter 8), will show that these effects are not related to any inhibition of ethanol metabolism by antioxidants. These results confirm the earlier findings of Rossiter and Slater (1973), who showed that antioxidants failed to reverse the increased NADH:NAD ratio in the liver after a large acute dose of ethanol (6 g/kg p.o.), yet prevented the hepatic triglyceride accumulation. Thus, the ability of (+)-catechin and 3-palmitoyl- (+)-catechin to prevent hepatic lipid accumulation after acute or
chronic ethanol administration are probably not related to these drugs' capacity to prevent the ethanol-induced redox-state changes, but rather to their antioxidant properties described earlier. The results thus cast some doubt on the popular 'redox-state theory' of fatty liver production discussed in detail in Chapter 1. Further experiments described here, confirm the potent ability of antioxidants to prevent fatty liver formation after large acute oral ethanol doses, and after chronic ethanol feeding.

The precise mechanism by which antioxidant substances might prevent fat accumulation is not clear from these experiments. However, one common factor between the findings with (+)-catechin, BHT and DPPD is reversal of the decreased ability of liver tissue to oxidise fatty acids after acute ethanol dosing. The studies reported in the previous chapter showed a good correlation between inhibition of fatty acid oxidation and increased fatty acid esterification into triglyceride in vitro, so the same mechanism may contribute to the process of ethanol-induced triglyceride accumulation in vivo. The protective effect of antioxidants against decreased lipid oxidation suggests that some peroxidative damage may occur to mitochondria in vivo, that affects fatty acid oxidation. This is possible, since lipid hydroperoxides have been observed to inhibit fatty acid oxidation in vitro (Imagawa et al, 1984).

An additional possibility is that some peroxidative event after ethanol dosing may limit hepatic triglyceride secretion, although the role of this mechanism in acute hepatic steatosis is disputed (Wallin et al, 1984). It seems unlikely that other effects of antioxidant
administration, such as impaired hepatic fatty acid uptake or decreased lipolysis are involved in the prevention of hepatic triglyceride disposal described here, since BHT and DPPD alone did not affect hepatic triglyceride levels, and previous studies have shown that administration of large doses of a variety of antioxidants, including DPPD, fails to influence peripheral lipolysis (Ugazio et al., 1973).

One of the major findings here is that standard synthetic antioxidants protect against ethanol hepatotoxicity under identical experimental conditions to those under which (+)-catechin and 3-palmitoyl-(+)-catechin have been shown to exert their protective effects. The lipid peroxidation measures in the chronic DPPD study showed that this substance did considerably improve the antioxidant status of the liver cell membranes, although ethanol feeding only caused a small, non-significant increase in this parameter. Previous studies have demonstrated an enhanced susceptibility of hepatic lipids to peroxidation after chronic ethanol feeding (MacDonald, 1973; Shaw et al., 1981; Roauch et al., 1983), using the more sensitive diene conjugation method.

Redox-state measurements were not made in the chronic experiment, but the findings described earlier, and the data obtained by Rossiter and Slater (1973) on the effect of antioxidants on the hepatic NADH:NAD ratio after acute ethanol dosing, indicate that the protective action of antioxidants observed in chronic ethanol feeding or after large acute oral doses of ethanol, is probably not due to
these substances affecting the ethanol-induced redox-state change. Work which has been reported in Chapter 6 indicates that hepatic lipid accumulation resulting from both acute and chronic ethanol feeding is probably independent of the increase in the NADH:NAD ratio caused by ethanol metabolism. Serum ethanol levels and ethanol intake data from the chronic DPPD experiment indicate that the protective effect of the antioxidant is not related to alterations in ethanol preference or metabolism. All of these considerations point to the antioxidant properties of BHT and DPPD being responsible for their protective effect on alcoholic fatty liver, and further suggests that it is similar properties of (+)-catechin and 3-palmitoyl-(+) catechin that confer protective capabilities on the latter compounds, rather than redox-state correction being their mechanism of action.
Chapter 8

THE EFFECTS OF 3-PALMITOYL-(+)-CATECHIN, NALOXONE AND ANTIOXIDANTS ON ETHANOL AND ACETALDEHYDE METABOLISM AFTER ACUTE ETHANOL ADMINISTRATION

8.1 INTRODUCTION

In Chapter 1 (section 1.2.2), the role of ethanol metabolism in the production of alcoholic fatty liver was discussed in detail. To summarise that discussion briefly, uninterrupted ethanol metabolism appears to be a requirement, certainly for the production of acute ethanol-induced fatty liver, and possibly also for the steatosis resulting from chronic ethanol administration. Thus, one mechanism by which drugs can reduce the extent of hepatic triglyceride accumulation is through interference with ethanol metabolism, as has been described for pyrazole (Morgan and DiLuzio, 1970). Thus, in this chapter, the effect of the agents investigated in the previous chapters on blood ethanol levels after an acute ethanol dose, under conditions identical to those used to study the redox-state changes and lipid utilisation, has been investigated to determine whether any of the effects of the compounds could have been due to alteration of ethanol metabolism.

It was also discussed in Chapter 1 (section 1.2.3) that there are probably two major primary metabolic consequences of ethanol
metabolism that could initiate the sequence of events that causes fat accumulation, one of these being the redox-state change, the other being production of acetaldehyde. The latter may cause triglyceride deposition through direct inhibitory effects on mitochondrial function (including fatty acid oxidation), impairment of lipoprotein secretion and/or initiation of peroxidative damage. Thus, the effects of 3-palmitoyl-(+)-catechin, Naloxone and antioxidants on hepatic acetaldehyde concentrations after acute ethanol dosing have been studied here, again, to determine whether any protective effects of these compounds (or lack of protection in the case of Naloxone) is due to alteration of hepatic steady-state levels of this ethanol metabolite.

8.2 METHODS

Male Wistar rats were used, and were fasted for 24 hours prior to ethanol administration. Ethanol was given (2 g/kg i.p. as a 20% (w/v) solution in saline) and 90 mins. later, animals were sacrificed by cervical dislocation and a portion of liver rapidly isolated by freeze-clamping. Hepatic acetaldehyde concentrations were determined in these frozen liver samples as described in section 2.6. Blood was collected from the necks of the animals and assayed for ethanol by head-space gas chromatography as described in section 2.6. Some animals were also sacrificed at three hours, or other time points after ethanol administration, in order to assess the effect of drug
treatments on blood ethanol and/or hepatic acetaldehyde concentrations at these various time points.

**Drug treatments:** Naloxone (2 mg/kg i.p.) was given at 1.0 h, and where appropriate, 2.5 h and 4.0 h after ethanol administration. The drug was given as pure Naloxone hydrochloride dissolved fresh in sterile saline, appropriate controls being sham-dosed with saline at the same time points. 3-Palmitoyl-(+)-catechin was suspended in 5% (w/v) Arabic Gum and given (100 mg/kg orally) at one hour before ethanol dosing. DPPD and BHT were given as emulsions in 0.5% (w/v) Gum Acacia containing 0.1% (w/v) Tween 80 at doses of 400 mg/kg (i.p.) at 24 and 1 hours before ethanol administration. Appropriate control animals were sham-dosed with vehicle substances at the same time points in all the experiments.

8.3 **RESULTS**

Hepatic acetaldehyde and blood ethanol concentrations after administration of ethanol in 3-palmitoyl-(+)-catechin-treated rats were determined at various time points after ethanol dosing. The drug had no influence on either parameter at all of the time points studied (Figs. 8.1 and 8.2). Since hepatic acetaldehyde concentrations appeared to reach an approximate steady-state value 90 mins. after ethanol administration in this experiment, in subsequent studies on Naloxone and antioxidants, acetaldehyde levels were studied at this time point only.
Fig. 8.1. EFFECT OF PALMITOYL-(+)-CATECHIN ON HEPATIC ACETALDEHYDE AFTER ETHANOL ADMINISTRATION (2 g/kg i.p.)

- **A** vs. **B**: N.S.

**Legend:**
- **●** = GROUP A (ETHANOL ONLY)
- **○** = GROUP B (ETHANOL + PALMITOYL-(+)-CATECHIN)

n = 4 rats per group per time point.
Results shown as means ± S.D.
Fig. 0.2. **EFFECT OF PALMITOYL-(+)-CATECHIN ON BLOOD ETHANOL AFTER ETHANOL ADMINISTRATION (2 g/kg i.p.)**
Naloxone administration also failed to affect either hepatic acetaldehyde or blood ethanol levels after acute ethanol administration (Table 8.1). Experiments with the antioxidants BHT and DPPD revealed that neither compound affected liver acetaldehyde levels, and DPPD also failed to influence the blood ethanol concentrations after this ethanol dose. BHT, on the other hand, appeared to cause a slight stimulation of ethanol elimination, as the blood ethanol concentration was significantly lower in animals treated with this antioxidant at three hours after ethanol administration (Table 8.2).

8.4 DISCUSSION

These experiments have shown that 3-palmitoyl- (+)-catechin does not affect hepatic acetaldehyde concentrations or the ethanol elimination rate after acute ethanol administration. Thus, the protective actions of the compound against the altered hepatic lipid disposal after ethanol dosing already described, cannot be attributed to either inhibition of ethanol metabolism (in a manner similar to pyrazole) or to 'trapping' of ethanol-derived acetaldehyde. The latter process has been proposed as the mechanism by which certain thiol compounds, such as cysteine, protect against ethanol hepatotoxicity (Hirayama et al, 1983).

Naloxone treatment also failed to affect ethanol elimination or acetaldehyde concentrations, indicating that correction of the
## TABLE 8.1

THE EFFECT OF NALOXONE ON HEPATIC ACETALDEHYDE AND BLOOD ETHANOL CONCENTRATIONS AFTER ACUTE ETHANOL ADMINISTRATION

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ethanol Only</th>
<th>Ethanol + Naloxone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic Acetaldehyde 90 mins after ethanol (nmol/g)</td>
<td>22.5 ± 4.1</td>
<td>27.8 ± 6.2</td>
</tr>
<tr>
<td>Blood Ethanol (mg/100 ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 h</td>
<td>219.8 ± 5.0</td>
<td>220.5 ± 5.0</td>
</tr>
<tr>
<td>3.0 h</td>
<td>139.9 ± 11.3</td>
<td>135.6 ± 11.1</td>
</tr>
<tr>
<td>6.0 h</td>
<td>72.3 ± 5.6</td>
<td>69.8 ± 10.8</td>
</tr>
</tbody>
</table>

Naloxone (2 mg/kg i.p.) was given at 1.0 hour, and where appropriate, 2.5h and 4.0h after ethanol (2.0 g/kg i.p.). Results are shown as mean ± S.D. (n = 6 rats per group). All differences are non-significant.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Ethanol Only</th>
<th>Ethanol + DPPD</th>
<th>Ethanol + BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Ethanol (mg/100 ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 h</td>
<td>22.5 ± 4.9</td>
<td>22.4 ± 2.6</td>
<td>24.9 ± 3.6</td>
</tr>
<tr>
<td>3.0 h</td>
<td>192.0 ± 10.0</td>
<td>205.0 ± 13.0</td>
<td>179.1 ± 20.9</td>
</tr>
<tr>
<td>1.5 h</td>
<td>139.9 ± 11.3</td>
<td>156.0 ± 22.1</td>
<td>117.3 ± 5.7*</td>
</tr>
</tbody>
</table>

DPPD or BHT was given (400 mg/kg i.p.) at 24 and 1 hour before ethanol administration (2.0 g/kg i.p.). Results are shown as mean ± S.D. n = 6 rats per group. * p < 0.005 vs. ethanol only group.
redox-state after ethanol both by this drug and 3-palmitoyl-(+)-catechin is not due to inhibition of alcohol dehydrogenase (ADH) activity. This being the case, it is thus possibly surprising that correction of the ethanol-induced redox-state change by either compound is not associated with any acceleration of ethanol metabolism. It has been proposed that the reoxidation of NADH, either to release ADH from inhibition, or to provide more NAD for the dehydrogenase, is a rate-limiting factor in ethanol oxidation (Badawy, 1978). However, the findings here do not support this hypothesis, and suggest that other factors, such as the hepatic acetaldehyde concentration during ethanol metabolism (Dawson, 1983), or the total hepatic ADH activity, may be of more importance in this respect.

The present data on the effect of Naloxone on blood ethanol concentrations does not agree with the findings of Badawy and Evans (1981) and Badawy and Aliyu (1984), both of these studies reporting lowering of blood ethanol concentrations by the drug in association with correction of the hepatic NADH:NAD ratio. These authors proposed that correction of the redox-state by Naloxone was the mechanism by which the blood ethanol concentration was decreased, assuming that the NADH:NAD ratio is important in regulating ethanol oxidation (Badawy, 1978). There has been much interest in the possible use of Naloxone as a treatment for acute alcohol-induced narcosis, some clinical studies demonstrating a 'sobering-up' effect of this opiate antagonist (Jeffcoate et al, 1979; Jeffreys et al,
1980). These clinical studies suggested that opioid receptors might be involved in the mechanism of ethanol intoxication, but Badawy and Evans (1981) subsequently presented a metabolic basis for the reversal of alcohol-induced narcosis as described above. Other clinical studies have failed to show any effects of Naloxone in alcohol intoxication (Nuotto et al, 1984) and the present results agree with those of Khanna et al (1982), who had to give Naloxone at doses of greater than 50 mg/kg i.p. before any effect on blood ethanol concentrations in mice could be observed. The discrepancy between the present data and that of Badawy and Evans (1981) and Badawy and Aliyu (1984) is difficult to account for, since identical ethanol doses, and a slightly higher Naloxone dose were employed. Differences in feeding status (the animals here were fasted, whereas those in Badawy's laboratory were fed) or strain differences in response to Naloxone are possible explanations.

The data obtained with the antioxidants BHT and DPPD also show that these compounds do not interfere with ethanol or acetaldehyde metabolism. Thus, it is feasible that the mechanism underlying the protective effects of these compounds described in earlier chapters, is scavenging of free radicals. BHT appeared to cause a slight increase in the rate of ethanol disappearance from blood, from the limited data presented here. This could be due to either an increase in microsomal ethanol-oxidising system (MEOS) activity resulting from induction of cytochrome P450 by the compound, or an increase in total liver ADH activity, since BHT is known to cause liver enlargement and stimulate hepatic mitotic activity (Lane and Lieber, 1967), in addition to being a microsomal enzyme inducer (Babich, 1982).
Chapter 9

EFFECTS OF 3-PALMITOYL-(+)-CATECHIN (3-PC) AND N,N'-DIPHENYL-P-
PHENYLENEDIAMINE (DPPD) ON HEPATIC LIPID PEROXIDATION AND
TRIGLYCERIDE CONTENT AFTER ACUTE ETHANOL DOSING

9.1 INTRODUCTION

The results of the experiments reported in the previous chapters are all consistent with free radical scavenging being the mechanism of action of the (+)-catechin compounds against ethanol-induced liver injury. In an attempt to provide some direct experimental evidence that the drugs may prevent steatosis at the same time as inhibiting ethanol-induced lipid peroxidation, the present set of experiments was carried out.

Some of the difficulties of determining hepatic lipid peroxidation after ethanol dosing, particularly in rats, have already been discussed in Chapter 1. Although, ideally, it would have been most appropriate to carry out studies on the effect of (+)-catechin on lipid peroxidation in the chronic ethanol feeding model, examination of some of the more recent studies on ethanol-induced lipid peroxidation (Macdonald, 1973; Torelli et al, 1978; Shaw et al, 1981) suggested that more consistent and reproducible results might be obtained using a moderately high (but not too high) acute oral dose of ethanol. Thus, in the current experiments, the effects of
3-palmitoyl-(+)-catechin, and one reference antioxidant compound (DPPD), on two parameters of lipid peroxidation (diene conjugation and malonaldehyde levels) have been determined at various time points after an acute oral dose of ethanol (5 g/kg), and these parameters related to the liver triglyceride and blood ethanol concentrations in the same experiments. It was hoped that this approach would indicate whether there is a causal link between ethanol-induced lipid peroxidation and triglyceride accumulation in the liver, and whether antioxidant compounds (including 3-palmitoyl-(+)-catechin) may prevent steatosis by inhibiting any lipid peroxidation observed under these conditions.

9.2 METHODS

Male Wistar albino rats (250-300 g) were used. They were maintained on a standard 41B cube diet and water, and were fasted for 24 hours prior to ethanol administration (5 g/kg p.o. as a 20% (w/v) solution in saline), control animals receiving an equivalent volume of saline by the same route. 3-Palmitoyl-(+)-catechin was given as a suspension in PEG 300 at 24 and 0 hours before ethanol, at a dose of 25 mg/kg (i.p.) on each occasion. DPPD was given in an identical manner at the same time points, but at a dose of 400 mg/kg i.p.

Animals were sacrificed at either 1.0, 3.0, 5.0. or 8.0 h after ethanol, and blood samples collected from the necks of the animals. Livers were rapidly excised, rinsed in ice-cold 0.25M sucrose buffered
with 10 mM potassium phosphate (pH = 7.4) containing 3 mM EDTA, blotted, weighed, and homogenised in the same medium to produce a 50% (w/v) homogenate. Portions of this freshly-prepared homogenate were immediately frozen in liquid nitrogen, and stored at -70°C for the analysis of reduced glutathione (GSH) and malonaldehyde (MDA) concentrations. Further portions of homogenate were diluted to 10% (w/v) with buffered sucrose-EDTA and centrifuged at 400 g for 10 mins to sediment the nuclei. The supernatent fraction was then centrifuged at 4000 g for 12 mins to produce a mitochondrial pellet. The supernatent from this last centrifugation step was then further centrifuged to produce a microsomal pellet as described previously. These subcellular fractions were assayed for diene conjugates, and malonaldehyde was measured in liver homogenates, using methodology described in Chapter 2 (section 2.9). Reduced glutathione (GSH) was measured in liver homogenates by the methyglyoxal method given in section 2.9.3. Blood ethanol was determined by head-space gas chromatography, and liver triglycerides measured by the method of Fletcher (1968), all experimental details for these assays being as given in Chapter 2.

9.3 RESULTS

Liver mitochondria and microsomes were examined for evidence of lipid peroxidation (as diene conjugate formation) at 1.0, 3.0 and 5.0 hours after ethanol dosing. At 3.0 h and 5.0 h after ethanol, there
was no evidence of increased diene conjugates in either subcellular fraction. At 1.0 h after ethanol, there was a significant increase in the absorbance at 233 nm of the mitochondrial lipids from ethanol-dosed animals, when values were compared with those found in saline-treated controls (Fig. 9.1), indicating the presence of diene conjugates. Repetition of this experiment confirmed the presence of this marker of lipid peroxidation in the liver mitochondria one hour after ethanol dosing, and also showed that pre-treatment with either 3-palmitoyl-(+)-catechin or DPPD largely prevented this peroxidative change caused by ethanol (Fig. 9.2). There was, however, no evidence of diene conjugate formation in the liver microsomes at 1.0 hour after ethanol dosing (Fig. 9.3). Treatment of animals with 3-palmitoyl-(+)-catechin or DPPD alone had no effect on the U.V. absorption spectra of the mitochondrial or microsomal lipids in control animals (results not shown).

Ethanol dosing caused significant increases in the liver malonaldehyde concentrations at 5.0 and 8.0 h after administration, both antioxidant compounds maintaining levels below control values in both ethanol-dosed (Fig. 9.4) and control animals (data not shown) throughout the period of study. The ethanol-induced decrease in liver GSH content was statistically significant by 5.0 h after dosing, this phenomenon not being reversed by treatment with either 3-palmitoyl-(+)-catechin or DPPD (Fig. 9.5). These compounds alone did not affect liver GSH concentrations in control animals (data not shown).
Absorbance / mg Lipid

Difference Spectrum
Ethanol vs. Control

Absorbance at 233 nm/mg Lipid
Ethanol: 0.699 ± 0.063
Control: 0.567 ± 0.037  p<0.01

Fig. 9.1. U.V. SPECTRUM OF MITOCHONDRIAL LIPIDS ONE HOUR AFTER ETHANOL (5 g/kg p.o.)
Fig. 9.2. U.V. SPECTRUM OF MITOCHONDRIAL LIPIDS ONE HOUR AFTER ETHANOL: EFFECT OF ANTIOXIDANTS
Fig. 9.3. U.V. SPECTRUM OF MICROSONAL LIPIDS ONE HOUR AFTER ETHANOL (5 g/kg p.o.)

Absorbance at 233 nm/mg Lipid

Control: 0.450 ± 0.040  N.S.
Ethanol: 0.451 ± 0.104  N.S.

n=4 rats/group
Fig. 9.4. LIVER MALONALDEHYDE CONCENTRATIONS AFTER ETHANOL (5 g/kg p.o.).

* = p < 0.02 vs. Controls and
** = p < 0.001 vs. Controls
+ = p < 0.01 vs. Ethanol only

- 227 -
Fig. 9.5. HEPATIC REDUCED GLUTATHIONE (GSH) LEVELS AFTER ACUTE ETHANOL ADMINISTRATION (5 g/kg p.o.)

![Graph showing the change in hepatic GSH levels after acute ethanol administration.](image)

- Control
- Ethanol only
- Ethanol + DPPD
- Ethanol + 3-PC

* = p < 0.01 vs Control
** = p < 0.005 vs Control

Hours after Ethanol:

1  2  3  4  5  6  7  8
Hepatic triglycerides were significantly increased, by 83% and 73% over control values, at 5.0 hours and 8.0 hours after ethanol dosing respectively. Treatment with either 3-palmitoyl-(-)-catechin or DPPD significantly reduced liver triglycerides in ethanol-dosed animals, both substances maintaining levels close to those found in control animals throughout the intoxication period (Fig. 9.6). 3-Palmitoyl-(-)-catechin and DPPD did not influence liver triglyceride concentrations in control animals (data not shown). Neither compound had any effect on blood ethanol concentration or the disappearance rate of ethanol from blood in ethanol-dosed animals (Fig. 9.7). Standard deviations have been omitted from the figures for clarity of presentation.

9.4 DISCUSSION

The experiments reported here have produced some limited evidence that acute ethanol dosing produces hepatic lipid peroxidation in the rat, in contrast to some earlier reports (Hashimoto and Recknagel, 1968). It is clear, however, that ethanol is a much weaker prooxidant than, for example, carbon tetrachloride. The diene conjugate changes in the mitochondria are in agreement with those reported by DiLuzio (1968) and MacDonald (1973), although it is interesting that the evidence of peroxidation here is rather transient, and is not detectable at any time in the microsomal fraction. It is difficult to assess the importance of this early peroxidative event in the
Fig. 9.6. HEPATIC TRIGLYCERIDES AFTER ACUTE ETHANOL ADMINISTRATION (5 g/kg p.o.)
Fig. 9.7. EFFECT OF ANTIOXIDANTS ON BLOOD ETHANOL CONCENTRATIONS AFTER ACUTE ETHANOL ADMINISTRATION (5 g/kg p.o.)
mitochondria to the hepatic triglyceride accumulation after ethanol, although it is interesting that this change precedes the peak in the liver triglyceride levels. The fact that 3-palmitoyl-(+)-catechin and DPPD inhibit the mitochondrial lipid peroxidation as well as preventing the increase in liver triglycerides after ethanol, suggests a causal link between these two phenomena. In addition, since 3-palmitoyl-(+)-catechin appears to exert almost identical effects in these experiments as DPPD, this further suggests that free radical scavenging is the mechanism of action of the (+)-catechin compounds.

The time-course of the changes in the liver malonaldehyde levels (or to be more precise, thiobarbituric acid (TBA)-reactive compounds), is rather different from the time-course of the changes in the mitochondrial diene conjugates. This may reflect the fact that these two factors occur at different stages in the peroxidative chain, or that the production of TBA-reactive material occurs as a result of ethanol-induced changes in the tissue (e.g.: triglyceride accumulation), rather than being a precursor lesion to any such effects of ethanol. Another possibility is that the increase in TBA-reactive material content of the liver increases after ethanol due to a decrease in the glutathione content of the tissue (Fig. 9.5). Interestingly, DPPD and 3-palmitoyl-(+) catechin do not prevent the ethanol-induced decrease in GSH concentrations, suggesting that the latter probably does not occur as a consequence of ethanol-induced lipid peroxidation.

It is clear from the blood ethanol data (Fig. 9.7), that DPPD and 3-palmitoyl-(+) catechin do not prevent peroxidation or triglyceride
accumulation after ethanol dosing through inhibiting ethanol metabolism in these experiments.

To conclude, the studies reported in this chapter have shown that there may be a causal link between lipid peroxidation and the hepatic triglyceride accumulation after acute ethanol dosing in rats, and further indicate that free radical scavenging could be the mechanism of action of the (+)-catechin compounds against ethanol hepatotoxicity, certainly in the acute situation. The results do not shed any further light, however, on the precise mechanism by which lipid peroxidation occurs after ethanol intake, or on the mechanism linking peroxidation to the observed triglyceride accumulation. Some of the possible mechanisms here have been discussed in detail in Chapter 1.
Chapter 10

CONCLUSIONS AND DISCUSSION

The aims of the work in this thesis were:

(i) To confirm and assess the potential protective properties of (+)-catechin and 3-palmitoyl-(+)-catechin against ethanol hepatotoxicity (fatty liver) in the rat.

(ii) To investigate the mechanisms underlying any such observed protective effects of these compounds.

The second of these aims is fairly difficult to achieve in the case of protective agents for alcoholic liver injury, since, as will be clear from the discussion in Chapter 1, there is much controversy as to what the important mechanisms causing the process are. For (+)-catechin, however, there were two possible candidates as potential mechanisms of action of the drug. Firstly, the correction of elevated NADH:NAD ratio (redox-state) in the liver after ethanol administration, and secondly, free-radical scavenging and the ability to inhibit lipid peroxidation, seemed feasible explanations for any protection of (+)-catechin against ethanol-induced steatosis in the rat.

In the first experiments, it was confirmed that (+)-catechin and also 3-palmitoyl-(+)-catechin, protected against both acute and chronic ethanol-induced fatty liver, possibly indicating that common mechanisms of action were operating in both experimental situations.
It has also been assumed that a common mechanism of action exists for both compounds studied, as discussed in earlier chapters. 3-Palmitoyl-(+)-catechin was more potent in the chronic ethanol-feeding model than the parent compound, probably as a result of its greater lipid solubility and more favourable pharmacokinetic characteristics. This work is the first demonstration of a protective action of the (+)-catechin compounds in a proper, controlled model of long-term alcohol feeding in the rat, previous studies employing a rather inadequate repeated ethanol-dosing regime to induce steatosis (Gajdos et al, 1972).

The first series of studies on the mechanism of action of the drugs, investigated the effect of the (+)-catechin compounds on the hepatic redox-state changes, and possible related components of fatty acid metabolism, so as to test the hypothesis of Gajdos et al (1972) that (+)-catechin acted through reversing the elevated NADH:NAD ratio after ethanol. Both (+)-catechin compounds were, in fact, found to correct the redox-state change in ethanol-dosed animals, at the same time as reversing the depressed fatty acid oxidation, and increased incorporation of fatty acids into triglycerides in the liver tissue, under these conditions of acute intoxication. This provided a fairly neat explanation for the way in which (+)-catechin might act, as well as apparently indicating that ethanol caused fat accumulation in the first place through a redox-state type of mechanism, suggesting a link between the NADH:NAD ratio change and the alterations in lipid metabolism.
To gain further evidence in favour of these proposed mechanisms, a series of experiments was carried out in which the hepatic redox-state was changed by means other than ethanol administration, namely, by giving sorbitol. This caused changes in the redox-state similar to those seen after ethanol, yet failed to affect the liver triglyceride content or lipid metabolism parameters, as studied above in the (+)-catechin experiments. Furthermore, amelioration of the ethanol-induced redox-state changes, either by Naloxone (in the acute study) or Methylene Blue (chronic study), failed to prevent the altered lipid metabolism or hepatic triglyceride accumulation. These findings obviously cast some doubt on the redox-state change as an important mechanism of fatty liver production, as well as showing that modulation of the NADH:NAD ratio by the (+)-catechin compounds has little part to play in the mechanism of protection of the drugs. For this reason, the possible mechanism by which (+)-catechin and 3-palmitoyl-(+)-catechin affects the NADH:NAD ratio was not pursued further, although it was evident that the compounds did not act through simply inhibiting ethanol metabolism.

Therefore, the remainder of the work investigated the possibility that the (+)-catechin compounds prevented fatty liver through acting as antioxidants, and preventing ethanol-induced lipid peroxidation. This was an opportunity to test the hypothesis of DiLuzio (1973) that lipid peroxidation was an important event in the pathogenesis of alcoholic fatty liver. Reference synthetic antioxidants (BHT and DPPD) were found to prevent the acute fatty liver, and DPPD prevented
the chronic ethanol-induced steatosis, under the same conditions as which the (+)-catechin compounds were found to be protective. In the acute experiments, antioxidants prevented fat accumulation and decreased fatty acid oxidation after ethanol without affecting the redox-state change, supporting the conclusions drawn from the earlier experiments that the increased NADH:NAD ratio is not responsible for the altered hepatic lipid disposal after ethanol. The ability of antioxidants to cause partial reversal of the reduced fatty acid oxidation in liver tissue after ethanol, suggests that peroxidative damage to the mitochondria may play a part in this phenomenon. This view is supported by data in the penultimate chapter showing that mitochondrial lipid peroxidation is an early event after acute ethanol dosing. These findings do not conclusively prove, however, that depressed lipid oxidation is the link between lipid peroxidation and triglyceride accumulation after ethanol.

Once it had been established that the (+)-catechin compounds and antioxidants did not prevent steatosis through interfering with ethanol metabolism or by altering steady-state acetaldehyde concentrations, an attempt was made to test the hypothesis that there was a causal link between lipid peroxidation and hepatic triglyceride accumulation after acute ethanol dosing, and that 3-palmitoyl-(+)-catechin prevented the latter process through inhibiting lipid peroxidation. It was found that the (+)-catechin compound, as well as DPPD, prevented the changes in lipid peroxidation parameters (mitochondrial diene conjugation and liver malonaldehyde)
at the same time as inhibiting triglyceride accumulation after acute ethanol administration. These findings support the DiLuzio (1973) hypothesis of alcoholic liver injury, as well as adding further evidence in favour of free radical scavenging being the mechanism of action of the (+)-catechin compounds.

In order to confirm that free radical scavenging is indeed the mechanism of action for (+)-catechin and 3-palmitoyl-(+)-catechin, further studies on the influence of these compounds on lipid peroxidation after chronic ethanol feeding could be performed. These would not be easy experiments to carry out, since ethanol is a fairly weak pro-oxidant and the changes in lipid peroxidation after chronic ethanol feeding in the rat are fairly subtle. It is unfortunate in this respect that ethanol is thought to interfere with one of the most sensitive methods for measuring in vivo lipid peroxidation, namely the measurement of exhaled hydrocarbons (ethane and pentane). Further experiments on the influence of a wider range of antioxidants (eg: propyl gallate or ethoxyquin) on chronic ethanol-induced fatty liver might clarify the role of lipid peroxidation in this situation. It is of interest, however, that throughout these studies, agents that have been found to protect against the acute triglyceride accumulation after ethanol also prevent the chronic fatty liver, suggesting similar mechanisms are at work in both experimental situations. The precise link between lipid peroxidation and fat accumulation after ethanol is still a matter for some speculation, however.
The findings presented here are certainly interesting in terms of re-evaluating the basic biochemical mechanisms of ethanol-induced liver injury. There is fairly good evidence that the elevated NADH:NAD ratio resulting from ethanol metabolism has little part to play in either the acute or chronic alcoholic fatty liver, which goes against a lot of conventional thinking in this area, this 'redox-state' mechanism being quite widely-quoted as the underlying cause of fat accumulation. Furthermore, these studies provide evidence in favour of the lipid peroxidation hypothesis of fatty liver production, so that future studies on ethanol hepatotoxicity should concentrate on this, and the possible role of acetalddehyde in the process, as discussed earlier. A simplified, diagrammatic representation of how ethanol metabolism may lead to hepatic lipid accumulation, and how the (+)-catechin compounds may interfere with the process on the basis of the work presented in this thesis, is shown in Fig. 10.1.

Finally, to put some of these findings into a clinical context, it has to be asked what the value, if any, of agents that prevent fatty liver is to patients with alcoholic liver disease. The arguments for and against fatty liver, a benign and reversible lesion though it is, being a precursor of the more severe forms of alcoholic liver disease (hepatitis and cirrhosis) have already been given in Chapter 1. If fat accumulation is important as a precursor event of cirrhosis, and it should be remembered that there is often a substantial degree of cell necrosis associated with fatty liver as
Fig. 10.1. POSSIBLE RELATIONSHIP BETWEEN ETHANOL METABOLISM, FATTY LIVER FORMATION, AND THE ACTION OF (+)-CATECHIN COMPOUNDS
well as haemodynamic alterations in the liver, preventing it may not be a bad thing, and could significantly affect the prognosis in those patients 'at risk' of proceeding to the full-blown cirrhotic lesion. Against this argument, however, there are a number of practical and ethical difficulties. Firstly, these protective agents are generally only effective prophylactically, and it would be very difficult to administer the substances to patients during ongoing alcohol consumption. Many patients do not present, of course, until they already have severe fatty liver or hepatitis. Secondly, there is the ethical problem of whether administering an agent that may limit liver damage to patients may, in fact, encourage them to drink more. If this was found to be the case, then, even though the liver may be protected, damage to other organs (pancreas or brain) may continue unabated and, of course, the drugs cannot overcome the sociological difficulties that arise out of frequent intoxication. Only closely-monitored clinical trials of these 'hepatoprotective' agents will provide answers to these questions.
REFERENCES


Treatment of alcohol-related liver disease with (+)-cyanidanol-3:
a randomised double-blind trial. Gut, 21, 965-969

vitro peroxidation of liver lipids in ethanol-treated rats.
Lipids, 8, 498-502

Conn, H.O. (1983). Cyanidanol: will a hepatotrophic drug from
Europe go West? Hepatology, 3, 121-123


by Isozyme 3a of liver microsomal cytochrome P-450.
Pharmacology, Biochemistry and Behaviour, 18, (Suppl.1), 177-180

bromotrichloromethane and ethanol acute intoxication. New
chemical evidence for lipid peroxidation in rat tissue microsomes.
Biochemical Journal, 212, 625-637

Role of acetate in the reduction of plasma free fatty acids
produced by ethanol in man. Journal of Lipid Research, 9,
509-512

Effect of ethanol consumption on the phospholipid composition of
rat liver microsomes and mitochondria. Biochimica Biophysica
Acta, 712, 225-233


Danni, O., Sawyer, B.C. and Slater, T.F. (1977). Effects of (+)-catechin in vitro and in vivo on disturbances produced in rat liver endoplasmic reticulum by carbon tetrachloride. Biochemical Society Transactions, 5, 1029-1032


Della Corte, E. and Stripe, F. (1972). The regulation of rat liver xanthine oxidase. Involvement of thiol groups in the conversion of the enzyme activity from dehydrogenase (type D) into oxidase (type 0) and purification of the enzyme. Biochemical Journal, 126, 739-745


Eliassen, K. and Osmundsen, H. (1984). Factors which may be significant regarding regulation of the clofibrate-dependent induction of the hepatic peroxisomal \( \beta \)-oxidation and hepatomegaly. Biochemical Pharmacology, 33, 1023-1031


Hashimoto, S. and Recknagel, R.O. (1968). No chemical evidence of hepatic lipid peroxidation in acute ethanol toxicity. Experimental and Molecular Pathology, 8, 225-242


Lossow, W.J. and Chaikoff, I.L. (1957). Carbohydrate sparing of fatty acid oxidation. I. The relation of fatty acid chain length to the degree of sparing. II. The mechanism by which carbohydrate spares the oxidation of palmitic acid. *Archives of Biochemistry and Biophysics, 57*, 23-40


Pieper, W.A. and Skeen, M.J. (1973). Changes in rate of ethanol elimination associated with chronic administration of ethanol to chimpanzees and rhesus monkeys. *Drug Metabolism and Disposition, 1*, 634-644


Rossiter, P. and Slater, T.F. (1973). The effects of antioxidants on the concentrations of reduced and oxidised nicotinamide adenine dinucleotide and of triglycerides in rat liver after the administration of ethanol. *Biochemical Society Transactions, 1*, 933-935


Saunders, J.B. and Williams, R. (1983). The genetics of alcoholism - is there an inherited susceptibility to alcohol-related problems? *Alcohol and Alcoholism*, 18, 189-218


*Biochemical Journal, 176*, 885-892


Schilling, R.J. and Reitz, R.C. (1980). A mechanism for ethanol-induced damage to liver mitochondrial structure and function. *Biochimica et Biophysica Acta, 603*, 266-277


Sorrell, M.F. and Tuma, D.J. (1978). Selective impairment of glyceroprotein metabolism by ethanol and acetaldehyde in rat liver slices. *Gastroenterology, 75*, 200-205


Tobon, F. and Mezey, E. (1971). Effect of ethanol administration on hepatic ethanol and drug metabolising activities and on rates of ethanol degradation. Journal of Laboratory and Clinical Medicine, 77, 110-121


Wooles, W.R. (1966). Depressed fatty acid oxidation as a factor in the etiology of acute ethanol-induced fatty liver. *Life Sciences, 5*, 267-276


Younes, M., Larseille, J. and Siegers, C.P. (1982). Effects of dithocarb and (+)-catechin on the glutathione conjugating system in rat liver cytosol *in vivo* and *in vitro*. *Pharmacological Research Communications*, 14, 779-788