Validation of a Liver Cell Model for Studies on the Effects of Some Micro-Food Components on Antioxidant Defense

A dissertation submitted for the degree of

Doctorate of Philosophy

By

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September, 1998

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Declaration

I hereby certify that the material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the context of the text.

Signed Helen Cantwell Date September 1998

Helen Cantwell 94971072
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Abstract

Validation of a liver cell model for studies on the effects of micro-food components on antioxidant defense.

Helen Cantwell, School of Biotechnology, Dublin City University, Dublin 9

The development of in vitro models for toxicity testing of food components is dependent on the availability of standardized methods of cell isolation, preparation and culture. Many groups isolate and use rat hepatocytes for a variety of purposes but the methods used for isolation vary greatly. Differences in isolation technique have been identified as a possible source of variation in the results obtained. This issue has led the European Centre for the Validation of Alternative Methods (ECVAM), following discussion with experts in the field of hepatocyte research, to recommend a standard hepatocyte isolation procedure. This project focused on the validation of a cell model consisting of short-term suspension cultures of rat hepatocytes isolated using the method recommended by ECVAM. Characterization of the hepatocytes with respect to structural and biochemical integrity and the retention of liver function was carried out via the assessment of dye uptake, cytosolic enzyme leakage, DNA, protein and ATP content, gluconeogenesis, choleseterogenesis and protein synthesis. Hepatocytes were found to be structurally and biochemically intact and to retain liver function. The effects of four micro-food components, 7-ketocholesterol, cholestanetriol, Cov1-ox and conjugated linoleic acid, on biochemical functionality and on the antioxidant defense system were then studied. The antioxidant defense system was studied through assessment of the extent of lipid peroxidation and the activity of the enzymes superoxide dismutase, catalase and glutathione peroxidase. 7-Ketocholesterol and cholestanetriol increased the activity of some or all of the antioxidant enzymes measured but did not induce lipid peroxidation. Coincubation of oxysterols with either Cov1-ox or CLA decreased the activity of the antioxidant enzymes to levels close to or below control. Lipid peroxidation in cells treated with CLA or Cov1-ox alone or in combination with 7-ketocholesterol or cholestanetriol was not found to be significantly different to control. This study provides indirect evidence that none of the test substances, at the concentrations studied, exerted prooxidant effects such as to induce lipid peroxidation in rat hepatocytes.
Publications and Presentations

Publications

Cantwell, H and Devery, R (1998) The response of the antioxidant defense system in rat hepatocytes is modified by Covi-ox Cell Biology and Toxicology 14 (6), p401-409


Cantwell, H., Pidgeon, G and Devery, R (1996) The effects of cholesterol 5α, 6α epoxide and cholestane 3β, 5α, 6β triol on lipid peroxidation and antioxidant enzyme responses in hepatocytes in suspension culture Cell Biology and Toxicology 13 (1) p33

Cantwell, H and Devery, R (1997) The effects of conjugated linoleic acid and Covi-ox on antioxidant enzymes in liver cells Irish Journal of Agricultural and Food Research 36 (2)


Cantwell, H., Devery, R, O’Shea, M and Stanton, C Isolated rat hepatocytes are a useful model for short-term studies of the effects of conjugated linoleic acid isomers on liver function Paper submitted to Lipids, September 1998

Oral Presentations


Posters


Cantwell, H. (1996). The effects of cholesterol 5α, 6α epoxide and cholestane 3β, 5α, 6β triol on lipid peroxidation and antioxidant enzyme responses in hepatocytes in suspension culture Poster presented at the International congress on hepatocytes, applications in cell biology, toxicology and medicine Physiologisch-Chemisches Institut der Universität Tübingen, Germany September 23rd – 26th 1996


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<tbody>
<tr>
<td>$\varepsilon$</td>
<td>Molar extinction coefficient</td>
</tr>
<tr>
<td>(β)NAD</td>
<td>$\beta$-Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>$\mu$Ci</td>
<td>Micro curies</td>
</tr>
<tr>
<td>ACO</td>
<td>Acyl CoA oxidase</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenine diphosphate hydrolysate</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenine monophosphate</td>
</tr>
<tr>
<td>Apo</td>
<td>Apoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyanisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSS</td>
<td>Balanced salt solution</td>
</tr>
<tr>
<td>CLA</td>
<td>Conjugated linoleic acid</td>
</tr>
<tr>
<td>Co A</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>COPs</td>
<td>Cholesterol oxidation products</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>Copper-zinc superoxide dismutase</td>
</tr>
<tr>
<td>DEAE-dextran</td>
<td>Diethylaminoethyl-dextran</td>
</tr>
<tr>
<td>DiI</td>
<td>3, 3’-Dioctadecylindocarbocyanine</td>
</tr>
<tr>
<td>DMBA</td>
<td>Dimethyl-benz[a]anthracene</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>DOTMA</td>
<td>N-[1,2,3-dioleyloxy)propyl]-N,N,N-trimethlyammonium chloride</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegrations per minute</td>
</tr>
<tr>
<td>ECVAM</td>
<td>European Center for the Validation of Alternative Methods</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGA</td>
<td>Ethylene glycol adipate</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EROD</td>
<td>Ethoxyresorufin-o-deethylase</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>FABP</td>
<td>Fatty acid binding protein</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detector</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GLC</td>
<td>Gas liquid chromatograph</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
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HDL  High density lipoprotein  
HEPES  N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]  
HMG Co A  Hydroxymethylglutaryl CoA  
H-TGL  Hepatic triacylglycerol lipase  
HUG  Hepatocyte User Group  
id  Internal diameter  
IDL  Intermediate density lipoprotein  
INT  2-(4-Iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride  
KRB  Krebs-Ringer bicarbonate  
l  Path length  
LDH  Lactate dehydrogenase  
LDL  Low density lipoprotein  
MDA  Malondialdehyde  
Mm  Minutes  
MnSOD  Manganese superoxide dismutase  
MNU  Methyl nitrosurea  
mRNA  Messenger ribonucleic acid  
MTT  3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, thiazolyl blue  
MUGT  Methylumbelliferyl UDP glucuronosyl transferase
<table>
<thead>
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<tr>
<td>mV</td>
<td>Millivolts</td>
</tr>
<tr>
<td>mw</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NADH</td>
<td>β-Nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide dinucleotide phosphate, oxidised form</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-Nicotinamide adenine dinucleotide phosphate, reduced form</td>
</tr>
<tr>
<td>NAR</td>
<td>Nagase analbuminemic rats</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>O₂</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>ODC</td>
<td>Ornithine decarboxylase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLPC</td>
<td>1-Palmitoyl-2-linoleoyl phosphatidylcholine</td>
</tr>
<tr>
<td>polyHEMA</td>
<td>Polyhydroxymethylmethacrylate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error mean</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
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<td>-----------</td>
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<tr>
<td>TCA cycle</td>
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<td>U</td>
<td>Units</td>
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<tr>
<td>UDPGT</td>
<td>Uridine diphosphate glucuronyltransferase</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V v v</td>
<td>Volume per volume per volume</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>V_s</td>
<td>Sample volume</td>
</tr>
<tr>
<td>V_t</td>
<td>Total volume</td>
</tr>
<tr>
<td>W/v</td>
<td>Weight per volume</td>
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<tr>
<td>WHHL</td>
<td>Watanabe Heritable Hyperlipidemic</td>
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CHAPTER 1

Introduction
11 Liver

Structure

In man, the liver is the largest internal organ weighing approximately 1.5 kg. It is situated in the abdominal cavity, see Fig 111, where it is attached to the diaphragm and protected by the ribs, see Fig 112. The human liver is a single organ although the different blood supplies to the liver have been used to divide the liver into lobes. In some animals, such as the rat, the liver is composed of lobes that are physically separate from each other. The liver is covered by a two-layered capsule. The outer smooth layer is composed of peritoneum whereas the inner layer, known as Glisson's capsule, is composed of a fibrous material which is also the structural material which supports the shape of the liver (Horner Andrews, 1979). The basic unit in the liver is the liver lobule which is cylindrical, several mm long and 0.8 to 2 mm in diameter. A diagrammatic representation of a liver lobule is shown in Fig 113. The lobule resembles a wheel with a vein at the center and hepatic plates as the spokes of the wheel. Hepatic plates consist of bile canaliculi flanked on each side by a layer of hepatocytes, one cell thick. These bile canaliculi empty into bile ducts. A sinusoid (a small blood vessel) separates each plate and is connected to the portal vein. These sinusoids carry portal blood to the hepatic plates. Unlike other organs, the liver has two blood supplies, portal and arterial. Oxygenated blood is carried from the hepatic artery, some supplies the septal tissue (tissue separating adjacent lobules) and some empties into the sinusoids. The sinusoids are lined with Kupffer cells and endothelial cells and these are separated from the hepatic plates by the Space of Disse (also known as the perisinusoidal space). This space prevents damage to the microvilli of hepatocytes by plasma contents that can pass.
Fig 111 Site of the human liver

The arrow indicates the liver
Fig. 1.1.2. Human liver, *in situ*

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through large pores present in the endothelial lining of the sinusoids. The Space of Disse is connected to lymphatic vessels and is involved in the drainage of excess fluid.

**Blood Supply**

As stated previously, the liver is unique in that it receives two blood supplies. Portal blood makes up about three-quarters of this supply (approximately 1100 ml per minute) and carries most oxygen and absorbed food to hepatocytes. If this supply is stopped leaving only the arterial supply, hepatocytes degenerate. The hepatic artery supplies the liver with about 350 ml of blood per minute. In the absence of stress, very little arterial blood is needed. However, if the arterial supply is stopped, death results. It is thought that this may be due to an increase in the concentrations of anaerobic bacteria following cessation of the supply of oxygenated blood, as administration of antibiotics can be life saving (Horner Andrews, 1979). The liver may also act as a blood reservoir. Normally it holds about 450 ml of blood but can expand if necessary and hold up to 1 litre. This can be necessary in the case of cardiac failure, where an increase in fluid retention and total body volume enhances venous return which may compensate for the heart’s diminished pumping ability (Guyton and Hall, 1996).

**Cells**

*Kupffer Cells* These are also known as reticuloendothelial cells and are not specific to the liver. They function as macrophages and cleanse the portal blood of the intestinal bacteria that it contains on entering the liver. They remove more than 99% of this bacteria and also old erythrocytes from which they liberate and release unconjugated bilirubin.
Fig. 1.1.3. Basic structure of a liver lobule.

Endothelial Cells

Hepatic endothelial cells are similar to other endothelial cells except for the presence of the pores. These pores are very large, up to 1 \( \mu \text{m} \), and allow the passage of plasma components into the Space of Disse. The presence of these pores means that there is a large quantity of lymph produced in the liver. Under resting conditions nearly 50% of lymph is produced by the liver. Hepatic lymph has a high protein concentration, almost as high as that of plasma.

Both endothelial and Kupffer cells contain cytochrome P450 enzymes and peroxidases and therefore can be involved in biotransformation. They also release mediators that regulate the function of hepatocytes and non-parenchymal cells.

Also connected with the sinusoid are fat storage cells, pit cells and bile duct epithelial cells. These sinusoidal cells make up about 30% of total liver cells and 20% of total liver volume.

Hepatocytes

These cells, which comprise about 70% of total liver cells and 60% of total liver volume, are also known as liver parenchymal cells, liver cells, and polygonal cells. These cells are usually smooth although their shape is variable. They have a larger proportion of mitochondria, lysosomes, and endoplasmic reticulum than most other cells.

An electrical potential exists across the cell membrane, the interior usually being \(-40 \text{ mV}\) with respect to the exterior. This resting potential can be altered by substances that affect glucose metabolism, such as adrenaline, noradrenaline, and glucagon.

All cells have slightly different properties depending on their position in the liver. Periportal cells receive most oxygen whereas those near the center of the lobe receive
At least periportal hepatocytes have higher levels of glutathione and a higher capacity for bile acid uptake while centrilobular hepatocytes have a higher concentration of cytochrome P450 enzymes. Hepatocytes seem to interact via humoral and nervous signals and also with the biomatrix in order to maintain glucose, ammonia and bicarbonate levels (Vickers, 1997)

**Functions of the Liver**

Hepatic cells are a large chemically reactant pool that have a high rate of metabolism, sharing substrates and energy from one metabolic system to another (Guyton and Hall, 1996). Metabolically, the liver is involved with carbohydrates, lipids and proteins. It is a storage site for carbohydrates, vitamins and iron. Some carbohydrates, proteins, lipoproteins, phospholipids and blood components are synthesized in the liver. It is the site of detoxification of drugs and toxins and also can contribute to body heat under extreme conditions. The liver therefore has very many varied functions.

**Carbohydrate Metabolism**

Saccharides, following ingestion are converted firstly to glucose, and then to glycogen for storage. Glycogen is a highly branched homopolymer of glucose with branches occurring every 8 to 10 glucose units. In this form glucose is stored in the liver until there is a decrease in the blood glucose level when glucagon will stimulate the breakdown of glycogen and therefore the release of glucose. In doing so, the liver ensures a constant blood glucose concentration and maintains a "glucose buffer function" (see Fig 114). Stored glycogen can maintain the blood glucose level for 24
Fig 1.1.4 The Glucose buffer function of the liver

Reproduced from Horner Andrews (1979), Liver
hours in the absence of food after which other sources, such as the breakdown of lipids, are employed (Horner Andrews, 1979) Gluconeogenesis may also occur This is the production of glucose from non-sugar components such as lactate or amino acids In the fed state, approximately 160 g glucose is required per day to supply tissues such as the brain and haemopoietic tissues The brain can adapt to using ketone bodies as an alternative energy source to glucose, during prolonged starvation In these cases the requirement for glucose is approximately 40 g per day (Souhami and Moxham, 1997)

Lipid Synthesis, Transport and Metabolism  A high rate of β oxidation of fatty acids occurs in the liver During this oxidation, which takes place mainly in the mitochondria, acetyl CoA is formed which becomes part of the tricarboxylic acid (TCA) cycle The liver cannot use all the acetyl CoA it produces and so it can be used for the production of ketone bodies This production takes place only in the mitochondrial matrix of liver cells and leads to the formation of acetoacetate, β-hydroxybutyrate and acetone Acetoacetate and β-hydroxybutyrate diffuse into the blood where they are transported to other tissues where they are converted back to acetyl CoA Liver cells do not possess one of the enzymes required to convert these ketone bodies back to acetyl CoA so the ketone bodies are produced in the liver but utilised elsewhere Ketone bodies are used to supply acetyl CoA for fatty acid and cholesterol synthesis and are an important source of energy for the brain during prolonged starvation (Guyton and Hall, 1996)

The first step in fatty acid synthesis is the conversion of acetyl CoA to malonyl CoA The acetyl CoA is provided by the liver as the end product of glycolysis or in the form of ketone bodies as explained above, assigning to the liver an important role in fatty
acid synthesis. Cholesterol is also synthesized in the liver. It is formed from acetyl CoA which is converted via hydroxymethylglutaryl coenzyme A (HMG CoA) to the isoprenoid precursor mevalonate. This is then converted via intermediates to squalene and finally to cholesterol. Cholesterol is then packed into lipoproteins for transport to other tissues.

Although the chylomicrons are synthesized in the intestine, all other lipoproteins originate in the liver. Very low density lipoproteins (VLDL) are formed in the endoplasmic reticulum (ER) of hepatocytes. The lipoprotein is globular with a core of hydrophobic lipids such as triacylglycerol or cholesterol ester surrounded by phospholipid proteins and cholesterol, oriented so that the polar parts are turned outwards and come in contact with the plasma. An apoprotein, formed in the ribosomes, is combined with the VLDL and then it is further processed in the Golgi complex where the apoprotein is glycosylated and the lipoprotein is membrane encapsulated to enable secretion into the plasma following fusion of the membrane capsule with the cellular membrane. The apoprotein that the newly secreted lipoprotein contains is apoB100. In the plasma the VLDL receives further apoproteins from high density lipoproteins (HDL) and starts to degrade when it receives apoCII. This protein can activate lipoprotein lipase which hydrolyses the core triglycerides to fatty acids and glycerol which are taken up by HDL. The size of the lipoprotein therefore starts to decrease leading to the formation of intermediate density lipoproteins (IDL). In humans the degradation of these lipoproteins continues to form low density lipoproteins (LDL) which deliver cholesterol to cells where it forms part of the cell membranes or is used as a precursor of steroid hormones. In species such as the rat however, this conversion to LDL does not occur and IDL are
removed by the hepatic remnant receptor HDL are also formed in the liver. This lipoprotein does not have a central lipid droplet but is thought to exist as a disc-like bilayer composed mainly of phospholipid and protein. The apoprotein associated with this lipoprotein is apoA1 (Vance and Vance, 1985).

Each day, approximately 0.5g cholesterol is converted into bile acids. In the liver, the two major bile acids — cholic acid and chenodeoxycholic acid are synthesized from cholesterol and conjugated to glycine or taurine. They are then secreted into the gall bladder and from there into the small intestine. The bile acids function in digestion by solubilising and emulsifying lipids. Bile acids are returned to the liver by an extremely efficient recycling system. However, a certain quantity, usually less than 1g a day, escapes the recycling system. This remains in the intestine where it is metabolised by microorganisms and excreted, representing the body's only mechanism for the excretion of cholesterol (Zubay, 1993). The role of the liver in lipid synthesis and metabolism is shown in Fig 1.1.5.

**Protein Synthesis and Metabolism** Amino acids must be deaminated before they can be used for energy or metabolised to another compound. This deamination takes place in the liver where the nitrogen is incorporated into urea via the urea cycle. The high concentrations of ammonia produced by the deamination of amino acids and the presence of intestinal bacteria from portal blood would lead to hepatic coma and death if not removed. Once deaminated, the carbon backbone of the amino acid can be converted to an intermediate of the TCA cycle and can then be degraded to carbon dioxide with the release of energy. The liver is the site of synthesis of approximately 90% of plasma
proteins, up to 15 to 20 grams per day. A decrease in the concentration of plasma proteins in the blood leads to growth of the liver and a rapid formation and excretion of plasma proteins until blood concentrations are back to normal.

**Blood Components and Vitamins** Many blood components are produced in the liver, particularly those involved in clotting. Fibrinogen, prothrombin, accelerator globulin, factors VII, VIII, IX and X are synthesised, for which vitamin K is required. Plasminogen, which is involved in liquefying clots, is also produced. To avoid the formation of blood clots in blood vessels, the liver removes clotting factors from the blood.

Vitamins A, B12, D, E and K are stored in the liver. The supply of vitamin A would last for 10 months whereas D would last 3 to 4 months and that of B12 would last for 1 to 3 years. Minerals such as iron, copper, zinc, cobalt, selenium, vanadium and molybdenum are also stored in the liver. Iron is stored in the form of ferritin. When there is a high concentration of iron in the blood, apoferritin, a hepatic protein, combines with the iron to form ferritin which it releases when the iron blood concentration is low. Another buffer function, that of a “blood iron buffer” is therefore assigned to the liver.

**Biotransformation Function** Excretion is the end-point for biotransformation so, to facilitate excretion by the kidneys or into the bile, more polar compounds are formed during biotransformation. The chemical structure of the parent compound is therefore changed which may effect its pharmacological and toxicological effects. Although
The role of the liver in lipid synthesis and metabolism.

biotransformation usually serves to detoxify, this is not always the case and polarity and water solubility may not always be increased.

Due to the high concentration of cytochrome P-450 enzymes and its position as a gateway to the other tissues of the body, the liver is the most important organ of biotransformation although other tissues such as the gut and lung may also be involved.

Biotransformation involves two types of reactions. The first type of reaction, known as phase 1, involves oxidation, reduction or hydrolysis of the parent compound. Usually this reaction introduces a functional group that will react with glucuronic acid, sulphate, glutathione or another highly polar compound during phase 2 reactions. This conjugation ensures the entire molecule is highly water soluble.

Phase 1 oxidations are catalysed mainly by the microsomal mono-oxygenases. This system is located in the smooth endoplasmic reticulum of cells and is based around the enzymes cytochrome P-450 and NADPH cytochrome P-450 reductase. There are different isozymes of cytochrome P-450 which are responsible for the oxidation of different substrates or for different types of oxidation of the same substrate (Timbrell, 1982). The cytochrome P-450 enzymes are haemoproteins that have an iron atom at the active site. In the Fe$^{3+}$ (oxidised) form, this iron atom binds the substrate. This enzyme-substrate complex is then reduced by NADPH cytochrome P-450 reductase before reacting with molecular oxygen and being reduced again. The complex splits into water, oxidised substrate and the oxidised form of the enzyme.

Oxidation reactions not carried out by microsomal enzymes include amine, purine, alcohol and aldehyde oxidation. Monoamine oxidase, a mitochondrial enzyme and diamine oxidase, a soluble enzyme, are involved in the deamination of primary,
secondary and tertiary amines such as putrescine. Purines seem to be oxidised by
xanthine oxidase and the most important enzyme for the oxidation of ethanol is alcohol
dehydrogenase, a soluble enzyme. Alcohol dehydrogenase converts an alcohol to the
corresponding aldehyde, which is in turn oxidised by aldehyde dehydrogenase.

The enzymes responsible for reduction reactions are found in the microsomal
fraction and the soluble cell fraction. Reduction reactions include the reduction of
aldehydes, ketones, double bonds and disulphides. The intestinal bacteria also play an
important role in the reduction of many azo compounds.

Esters and amides are usually hydrolysed, by enzymes usually found in the
soluble part of the cell. Epoxides are metabolised by epoxide hydratase. This enzyme is
located close to the cytochrome P-450 group of enzymes where it detoxifies the epoxide
products of cytochrome P-450 mediated hydroxylation.

Phase 2 reactions involve the addition of polar groups to the parent compound or
to the metabolite formed during phase 1 reactions. The entire molecule is therefore more
polar and more readily excreted. The polar groups added include glucuronic acid,
sulphate, glutathione, amino acids and acetyl and methyl groups. In some cases, the final
metabolite is more toxic than the parent compound, e.g. the glucuronide conjugate of
acetylaminofluorene is a more potent carcinogen than the parent compound.

1.2 Liver in *In Vitro* Research

Due to the very diverse biochemical functions of the liver and to its high
concentration of drug metabolising enzymes, the liver has been extensively used in
research. Whole organs, tissue slices, hepatocytes and subcellular fractions have been
employed. Although there are disadvantages of *in vitro* systems such as the difficulty of culturing or maintaining liver tissue and the fact that the *in vitro* system may not always be representative of mature, differentiated organs, *in vitro* systems hold many advantages. As well as the ethical considerations, by using an *in vitro* system, studies can be carried out at a cellular and subcellular level. Experimental conditions are known and the effects of a parent compound or a metabolite can be studied by controlling the presence or absence of liver S9 metabolising fractions. The presence of the S9 fraction would mean metabolism of the parent compound would occur and therefore effects of the metabolite could be studied. Absence of the S9 fraction means that metabolism of the compound does not occur and effects of the parent compound only can be studied. *In vitro* systems are also more time and labour efficient.

*Perfused Whole Organ*

When the whole liver is used, it is usually used in a perfusion system where the recirculating medium contains test substances and the medium emerging from the liver is tested. Marques-Vidal *et al.* (1994) used such a system to study the role of hepatic triacylglycerol lipase (H-TGL) in promoting the uptake of HDL cholesterol and HDL cholesteryl ester. In a recirculating system such as the perfused whole liver, the enzyme is physiologically expressed and active at the vascular bed. Male Wistar rats were anesthetized with sodium pentobarbital and an *in situ* liver perfusion set up. Following a pre-perfusion with Hank’s Balanced Salt Solution, a recirculating perfusion was initiated using 60-80 ml of fresh medium supplemented with 1% BSA (w/v), 5 mM glucose and HDL particles (100 μg/ml) which had been labeled with $^{14}$C cholesterol and $^3$H.
cholesteryl ether (a non-hydrolyzable tracer of cholesteryl ester) Temperature was maintained at 37°C and the flow rate was 10 ml/min. 1 ml aliquots of the perfusate were removed at various time intervals and measured for radioactivity. H-TGL was found to promote the uptake of HDL esterified cholesterol (Marques-Vidal, 1994). Mackinnon et al. (1986) used the perfused whole liver to study the role of the liver in the catabolism of HDL. Male English short-hair rabbits were anesthetized using pentobarbital. A pre-perfusion with Krebs-Ringer bicarbonate (KRB) solution was carried out before a recirculating perfusion, using KRB supplemented with 0.15% (w/v) glucose and 125I-labeled or 3H-labeled rabbit HDL at a concentration of 10-200% of the normal physiological concentrations of HDL in rabbit plasma (72 mg/dl). 100 μl aliquots of the perfusate were removed at 5 min intervals and measured for radioactivity. The study showed that uptake of HDL cholesteryl ester by the liver occurs more rapidly than uptake of HDL protein and that the uptake resulted in smaller HDL particles significantly depleted of core cholesteryl esters (Mackinnon et al., 1986).

Liver Slices

Studies carried out with liver slices are usually of short duration although slices may be maintained in culture for a number of days. As with liver cells, there is no standardised method for the preparation and maintenance of liver slices and both perfused and nonperfused tissue from fed and fasted animals has been used. (Lake et al., 1995) Various incubation temperatures and media have been used and different procedures for tissue coring and embedding have been described. The use of tissue slices increased following the development of the Krumdieck Tissue Sheer and have been used to
compare the metabolism of cyclosporine A in different species (Vickers, 1992). Species differences in coumarm metabolism to 7-hydroxycoumarin have been described (Steensma, 1994). Tissue slices have also been used to identify focal areas of injury by monitoring histopathological changes, (Ruegg, 1985) and to investigate the genotoxic potency of the steroid hormones chlormadinone acetate, cyproterone acetate and megasterol acetate by examining for the production of DNA adducts, (Baumann et al., 1995). Baumann et al. (1995) suggested that the preparation of human tissue slices is less complicated than that of human hepatocytes. Many studies have been carried out using both tissue slices and isolated hepatocytes and the results using the two methods compared. One such study was carried out by Ekins et al. in 1995, investigating the metabolism of various hormones. They concluded that, although hepatic architecture was maintained in the liver slices, the rate of uptake seems to be restricted compared to isolated hepatocytes and suggest that it is likely that only the outer two layers of cells either side of the slice are responsible for metabolism during incubations of up to 1 hour. Howells et al. (1995) compared liver slices and hepatocyte monolayers with respect to the levels of the xenobiotic metabolising enzymes ethoxyresorufin-o-deethylase (EROD) and methylumbelliferyl UDP glucuronosyl-transferase (MUGT). The activities of both EROD and MUGT were found to be consistently higher in hepatocyte monolayers than in liver slices.

Isolated Hepatocytes

The use of isolated hepatocytes, either in suspension culture, monolayer culture or in some form of three-dimensional culture system has been extensively described in the
As xenobiotics ingested orally tend to pass exclusively into the hepatic portal vein following absorption in the stomach and intestines, hepatocytes have been used to study the effect of many substances found in the diet. The toxic effects of by-products of drinking water chlorination (e.g., chloroform and chloroacetates) have been studied. The effects of chloroform were studied by El-shenawy and Abdel-Rahman (1993) using biomarkers such as leakage of the enzymes aspartate transaminase and alanine transaminase and also levels of glutathione, glutathione peroxidase and glutathione reductase, substances involved in antioxidant defense. Mono-, di- and trichloroacetate, by-products of drinking water chlorination, were investigated for their toxic effects using hepatocytes, (Bruschi and Bull, 1993). The garlic extracts, allicin and ajoene, were found to inhibit the synthesis of cholesterol, (Gebhardt et al., 1994). Hepatocytes isolated from male Sprague Dawley rats using a two-step perfusion procedure were cultured on a layer of collagen in 2 ml Williams E medium containing 10% newborn calf serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.1 μM dexamethasone. The cells were then incubated at 37°C, 90% humidity and 7% CO₂. After two hours of incubation the medium was replaced and serum-free medium was used for further incubation. 5 mM allicin and 10 mM ajoene were prepared in Williams E medium (serum free) and added to the cell cultures to give a final concentration in the range of 10⁻⁶ and 10⁻³ M. Microsomes were prepared and HMG CoA reductase activity determined by measuring the conversion of ¹⁴C HMG CoA to ¹⁴C mevalonate. Both compounds were found to induce a dose-dependent decrease in cholesterol biosynthesis (Gebhardt et al., 1994). α-Amanitin and dl-propargylglycine from the poisonous mushroom Amanita abrupta, although inducing a slight increase in lipid peroxidation, did not seem to be
responsible for the toxic effects of the mushroom seen in vivo, (Kawaji et al, 1990) Contaminants of food substances have also been investigated Ichikawa et al (1996) used the measurement of albumin secretion in hepatocytes to determine the long term cytotoxicities of pesticides and Ohtani et al (1996) examined the effect of the cholesterol oxide 7-ketocholesterol on hepatocyte viability and lipid peroxidation

The liver is an ideal site to examine for possible side effects of orally ingested drugs Rodriguez et al (1995) studied the antifungal agents, fluconazole and ketoconazole (also an anticancer agent used in the treatment of prostate cancer) Using microscopy, LDH leakage and the neutral red and MTT assays, it was found that ketoconazole was more hepatotoxic than fluconazole

Hepatocytes have also been used in basic research to elucidate biochemical pathways and to examine basic cellular functions Zaleski et al (1977) found that the fatty acids oleate, palmitate and octanoate are potent regulatory factors of both the rate of glucose formation and the contribution of mitochondrial phosphoenolpyruvate carboxykinase to gluconeogenesis Luo et al (1995) found that, provided with a variety of substrates, sheep hepatocytes preferentially incorporate the amide nitrogen of glutamine in urea synthesis and Vericel et al (1994) studied the effects of age and hypertension on the antioxidant defense system and also on lipid peroxidation using hepatocytes Hepatic antioxidant defense was found to decrease with advancing age and glutathione peroxidase activity was found to be lower in hypertensive rats than in normotensive rats Unfortunately, liver function is in some ways dependent on its anatomy and the organ architecture is lost when isolated hepatocytes are used Oxygen concentration gradients
are therefore lost and the absence of liver lobes means that the polarity of the hepatocyte is also absent which largely prevents biliary secretion of drugs (Berry et al, 1991)

Subcellular Fractions

Hepatic subcellular fractions have been used in research. Rakowska et al (1997) reported the use of isolated hepatic endoplasmic reticulum membranes to study the incorporation of aminoalcohols into phospholipids. Isolated liver mitochondria were used to study the mitochondrial antioxidant defense system. (Augustin et al, 1997) Microsomes have been used to study antioxidant function. In 1990 Kagan et al studied the effect of chain length on the antioxidant function of tocopherol homologues using rat liver microsomes and Pulla-Reddy and Lokesh (1992) used a similar system to study the antioxidant effects of spice components such as curcumin, capsaicin and piperine. For much drug metabolism research, hepatocytes have the advantage that microsomes may not possess the complete drug metabolising sequence and therefore for overall metabolism of a compound it would be necessary to employ hepatocytes as the research tool. However, for specific components of xenobiotic metabolism microsomes may be suitable. For example, Burke et al (1985) used liver microsomes to discover the specificity of cytochrome P450 isozymes for different substrates.

1.3 The Liver and Gene Technology

Genetic defects are responsible for many liver-based diseases including Familial Hypercholesterolemia, Wolman’s disease and hyperlipoproteinemia. Recovery from such
diseases can only occur when such defects have been identified and corrected. In recent years, much research has been dedicated to the development of a method which will allow the introduction of corrective DNA and its permanent expression. Both *in vivo* methods (the injection of DNA, packaged in some form, into the host) and *ex vivo* methods (the removal of the target cells, introduction of the DNA and the transplantation of the modified cells back into the host) have been investigated. The methods of delivering the DNA include the use of retroviral vectors, adenoviral vectors, cationic liposomes and polymer gels.

Retroviral vectors have the potential to produce permanent gene expression through integration of the viral vector into the host DNA. However, it is necessary for the target cells to proliferate so that this integration may occur. Hepatocytes, the target cells in the treatment of most of the aforementioned diseases, are usually non-proliferative and so the use of retroviral vectors produces transient gene expression only. Adenoviral vectors can infect non-proliferative cells as they do not integrate into the host DNA but remain in a stable extra-chromosomal form. High percentages, approximately 100%, of cells may be transfected but again expression is transient. Use of both retro- and adenoviral vectors can elicit an immune response (Nabel, 1995).

Cationic liposomes are formed by the spontaneous reaction of a reagent, such as DOTMA (N-[1-2,3-dioleyloxy)propyl]-N,N,N-trimethyammonium chloride), with DNA to form lipid-DNA complexes due to the ionic interactions between the positive groups on the DOTMA and the negative groups on the DNA. This method leads to a high transfection efficiency but high levels of the lipids and exposure to the lipid over a moderate period of time is toxic.
The most efficient in vivo method of transferring DNA developed so far seems to be the use of a retroviral vector while stimulating the proliferation of hepatocytes. This can be accomplished by partial hepatectomy and was employed for the treatment of diabetes mellitus in rats (Kolodka et al., 1995). Diabetes mellitus occurs when pancreatic β cells are destroyed and so insulin production is arrested. It has been observed that liver will produce insulin on the introduction of the appropriate gene. After 70% partial hepatectomy and treatment with a retroviral vector, expression of the gene was seen for at least 6 months. Although the proinsulin produced was not completely processed and so was less biologically active, none of the conditions normally associated with severe diabetes occurred. A major drawback of this procedure is that diabetes was induced two weeks after the introduction of the DNA. The reason for this was that due to altered liver regeneration kinetics of severely diabetic rats, the efficiency of transfer was very low.

A method for inducing hepatocyte proliferation which does not involve hepatectomy has been developed by Lieber et al., (1995). A combination of adenovirus and retrovirus was used. The adenovirus, incorporating the urokinase gene, infected 90% of the hepatocytes, causing expression of urokinase and so stimulated necrosis in these cells. Retrovirus was then introduced and incorporated into the genome of the hepatocytes which were now proliferating. The hepatocytes continued to proliferate until, after a period of three to four weeks, the liver appeared normal again. This allowed for multiple infusions of retrovirus which, when carried out on mice, yielded gene expression five times greater than that seen using hepatectomy to stimulate proliferation. The authors suggest that the genetic effect is permanent.
Ex vivo gene therapy, i.e., the isolation of, in this case, hepatocytes, their transformation in culture and the reintroduction into the host has also been studied. The Watanabe Heritable Hyperlipidemic (WHHL) rabbit was subjected to a 30% partial hepatectomy and the hepatocytes isolated. The hepatocytes were exposed to a retrovirus incorporating the LDL receptor gene and then reharvested. These modified cells were injected into the spleen of the animal. A decrease of between 25 and 45% in serum cholesterol was seen within two days and lasted for six and a half months (the duration of the experiment) (Wilson et al., 1990).

Rapper et al. (1992) used retrovirus to introduce genes encoding β-galactosidase and the LDL receptor into human hepatocytes. β-galactosidase expression was found in 43.7% of cells incubated with the retrovirus Dil-LDL (LDL particles labeled with the fluorimetric component 3,3'-dioctadecylindocarbocyanine) uptake was used to determine expression of the LDL receptor gene and the level of expression was found to be dependent on plating density.

Watanabe et al. (1994) studied the use of cationic liposomes of various composition and on hepatocytes from different species. After examining DEAE-Dextran, calcium phosphate, cation liposomes and cation multilamellar liposomes, they achieved 60% transfection rates but mention that the ratio of DNA to reagent and the preincubation of DNA and reagent to form complexes are critical and also that high cell density and the presence of serum decrease the transfection rate. The 60% transfection rates were achieved in mouse and hamster but not in rat hepatocytes. Watanabe et al. (1994) also conclude that the uptake of liposomes occurs by endocytosis. The inhibition of
transfection by free LDL, EDTA (removes calcium necessary for endocytosis) and cytochalasin B, an endocytosis inhibitor, led to this conclusion.

The effective ex vivo transduction of hepatocytes would be very beneficial as according to Gupta et al. (1995) transplanted hepatocytes are incorporated into liver plates, developing gap junctions, bile canaliculi and retaining liver specific functions.

1.4 Liver Assist Device

The possibility of using hepatocytes as part of a liver assist device has attracted a lot of attention in recent years. Hepatocytes may be directly transplanted or used in a hybrid bioartificial form. The advantages of direct transplantation are that hepatocytes should carry out all the functions of an intact functional liver but small amounts can be donated by relatives or one whole liver can supply many patients. Direct infusion of hepatocytes into the portal vein causes the formation of aggregates and so induces necrosis. Many other sites have been suggested as possible infusion sites but the only two that seem promising are the spleen and the peritoneal cavity. The latter means a simpler transplantation procedure and a larger quantity of hepatocytes transplanted, but many researchers advocate use of the spleen as hepatocytes have been shown to survive for a year and have differentiated function (Wang, 1995).

Hybrid bioartificial devices have both a biological and a synthetic component. As part of a hybrid bioartificial unit, hepatocytes have been used:

- With microcarriers
- In a microencapsulated form
- With a scaffold

25
As part of a bioreactor

**Microcarriers**

These are made from collagen-coated dextran or more recently polyHEMA (polyhydroxymethylmethacrylate). These microcarriers have a large surface area to volume relationship and so large quantities of hepatocytes can be present in small volumes. Demetriou *et al.* (1986) successfully used microcarriers to treat Gunn rats which have an inherited deficiency of UDPGT (bilirubin-uridine diphosphate glucuronosyltransferase) and so cannot conjugate bilirubin and also to treat Nagase analbuminemia rats (NAR) which cannot produce albumin. On examination, it was found that the injected microcarriers had formed aggregates in the peritoneal cavity, within which new blood vessels had begun to form. The transplantation did not seem to be toxic in any way but optimum results were achieved when immunosuppressants were used. Fig 14.1 shows a diagrammatic representation of microcarriers.

**Microencapsulation**

Microencapsulation means the hepatocytes are immobilised in a 3-D collagen matrix that may promote differentiated function. The hepatocytes are encapsulated in an ultra-thin, semi-permeable membrane, which, while allowing nutrient and gas exchange pertaining to biological function, protects the hepatocytes from components of the immune system (Dixit, 1995). Microencapsulated hepatocytes are shown in Fig 14.2. Wang (1995) advocates “resuscitating” hepatocytes prior to transplantation. This involves culturing the hepatocytes for three days with a hepatotrophic factor such as
Fig 14.1 Hepatocytes immobilised on microcarriers

Reproduced from Dixit (1995)
Fig. 14.2 Microencapsulated hepatocytes

Reproduced from Dixit (1995)
insulin Hepatocytes would therefore have time to recover from the trauma suffered during isolation and become fully functional again

Scaffold

Cima et al (1991) had come to this same conclusion when they described the development of a scaffold which would be used for hepatocyte transplantation. After isolation of hepatocytes, the cells are cultured on this scaffold for three to five days. This leaves time for the cells to recover and also extensive viability and functional assays to be carried out. At the end of this time period the entire structure may be transplanted. Collagen, which is typically used for hepatocyte culture has poor mechanical stability and so is unsuitable for the construction of such a device. Cima et al (1991) compared polylactic acid, polyglycolic acid, poly-co-glycolic acid and different blends of these to find the optimal material with which to construct the scaffold. Promising results were obtained as hepatocytes cultured on these polymers maintained differentiated function and were found to have better cell attachment rates than with collagen.

Bioreactors

The use of an extracorporeal device stems from 1958 when Kiley et al described using Kolff hemodialysis to treat five patients, four of which showed an improvement. Since then such liver support has ranged from canine and human cross circulation, perfusion through livers from various species, charcoal hemoperfusion and blood and plasma exchange. Four types of modern bioreactors are described here.
Plate Dialyzers  Multiple plates of hepatocyte monolayer cultures are arranged so that blood flows over the side of the plates opposite to that in which the hepatocytes are cultured (Dixit, 1995) Very little has been published about this type of reactor

Hepatocyte Hemoperfusion  This involves the direct perfusion of blood over hepatocyte cultures The hepatocytes may be microencapsulated to avoid immune reactions Because of the resulting high surface area to volume ratio, maximum nutrient/product exchange occurs It is thought that current microcapsules may not be strong enough to withstand stresses accompanying high velocity flow rates (Dixit, 1995) In cats, this type of bioreactor was capable of replacing liver function for up to 4 hours (Yanagi and Ookawa, 1989)

Packed Bed Bioreactor  This bioreactor is composed of a glass vessel packed with glass beads A hepatocyte suspension is recirculated through the vessel until attachment occurs Li et al (1993) found that aggregates had formed within the spaces between the beads and that these aggregates were all interconnected by “cords” which had also formed The cells were described as cuboidal in shape, had organelles, desmosomes, glycogen granules and peroxisomes The hepatocytes were examined for up to fifteen days and remained viable for this period

Capillary Hollow Fiber Hepatocyte Bioreactor  The basic form of this reactor consists of semipermeable capillary hollow fiber membranes enclosed in a plastic shell Hepatocytes are cultured on the exterior of the membranes The membranes are perfused
with blood. The hepatocytes perform liver functions and are protected from the immune system by the membranes.

Modifications to this basic structure include the use of microcarriers with which to attach the hepatocytes, the entrapment of the hepatocytes in a 3-D collagen gel inside the hollow fibers- this allows the hepatocytes to be cultured in a 3-D matrix but still be protected from the immune system as culture medium is perfused through the fibers and blood through the space outside the fibers (Shatford et al, 1992) Finally, a system which closely resembles the cellular organisation of the liver was described by Dixit in 1995. Two different types of capillary hollow fibers are used. Hepatocytes are attached to the external surface of one through which a medium flows. The other has no hepatocytes but the blood is perfused through these in a counter current to the nutrient flow. The toxins and metabolites produced during liver disease diffuse out of the capillaries and are carried on a concentration gradient to the hepatocytes where they are acted on and the end products are removed via the nutrient stream. Essential proteins etc. produced by the hepatocytes are again carried via a concentration gradient this time to the fibers containing the blood into which they diffuse. A diagrammatic representation of this type of bioreactor is shown in Fig. 143.

Demetriou et al (1995) present clinical data that shows promising results using a bioartificial liver as a bridge to organ transplant. The device they used consists of a hollow fiber bioreactor as described onto which porcine hepatocytes are cultured using
Fig 14.3 Capillary hollow fiber hepatocyte bioreactor

Reproduced from Dixit (1995)
Before being introduced to the bioreactor the plasma is passed through an activated cellulose coated charcoal column to protect the hepatocytes from the possible toxic effects of hepatic failure plasma.

Following transplantation, graft dysfunction occurs in about 10% of patients and survival depends on early retransplant. The aetiology has yet to be defined but reperfusion injury as a result of free radical generation has been considered as a possible mechanism (Toleda-Perevra, 1991, Bzeizi et al, 1992, Connor et al, 1994). The pharmacological manipulation of reperfusion injury will require the use of various substances that can protect the transplanted liver from the formation of free radicals. Among these, free radical scavenger enzymes such as superoxide dismutase have been shown to exert beneficial effects on liver grafts (Mizuta et al, 1989, Minor et al, 1992). The remainder of this chapter will focus on the chemistry of the major free radical species encountered in cellular systems, the mechanism of free-radical mediated lipid peroxidation and the protective effects of antioxidants and free radical scavenging molecules.

1.5 Free Radicals and Oxidation

Halliwell and Gutteridge (1989) define a free radical as “any species capable of independent existence that contains one or more unpaired electrons.” Free radical sources in the environment include air pollutants, insecticides, herbicides and some drugs. Biologically, free radicals are formed from polyunsaturated fatty acids (PUFA), sulphhydryl compounds and quinones or quinone-like substances. The most important source of radical species in biological systems, however, is O₂ (Singal et al, 1988). At
53% oxygen is the most prevalent element in the earth's crust and, although essential for life, at concentrations greater than that found in air it is known to be toxic to plants, animals and bacteria. There is also some evidence that at 21% there exists some slowly manifested effects of oxygen (Halliwell and Gutteridge, 1989). The toxic effects of oxygen include the direct or indirect inhibition of enzymes and the production of free radicals, the toxic effects of which are shown in Fig. 15.1

**Free Radical Formation**

Free radicals are formed through homolytic fission, which is the breaking of a covalent bond where one electron from each pair shared remains with each atom.

\[ A_x^xB \rightarrow A^x + B_x \]

Heterolytic fission, where both electrons remain with one atom, results in ion formation:

\[ A_x^xB \rightarrow A_x^x + B^+ \]

Oxygen readily accepts electrons from other substances – it is a good oxidising agent, but tends to accept electrons one at a time to comply with spin restrictions. Oxygen therefore reacts sluggishly with non-radicals. The spin restriction is removed in singlet oxygen (ground state oxygen has one each of its outer unpaired electrons in the \( \pi^* \) and \( \sigma^* \) orbitals whereas in singlet oxygen both electrons are in the \( \pi^* \) orbital) and so it has increased reactivity. There are no unpaired electrons however so it is not a radical. It can be formed in some radical reactions, however, and can trigger others. Singlet oxygen yields endoperoxides on reaction with conjugated double bonds, hydroperoxides on reaction.
with double bonds and carbonyl compounds on reaction with a double bond in conjugation with an electron donating atom such as nitrogen or sulphur. These reactions are shown below.

\[
\begin{align*}
\text{Reaction with conjugated double bond to form an endoperoxide} \\
\text{Reaction with a double bond to form a hydroperoxide} \\
\text{Reaction with a double bond adjacent to an electron-donating atom to form carbonyl compounds via a dioxetane intermediate}
\end{align*}
\]
Fig. 1.5.1 Reactive oxygen species and cell injury

The addition of one electron to ground state oxygen generates the superoxide radical

\[ \text{O}_2 + e^- \rightarrow \text{O}_2^- \]

It has one unpaired electron and is therefore less of a radical than oxygen. It shall henceforth be denoted by \( \text{O}_2^- \). It is stable in both aqueous and organic environments although it’s chemical properties differ in each. In aqueous solution it can accept a proton and form the hydroperoxy radical \( \text{HO}_2^- \) but at the pH of body fluids most remains as \( \text{O}_2^- \).

It can also undergo a dismutation reaction that results in the formation of hydrogen peroxide. It can act as both a reducing agent and a weak oxidising agent (Davies, 1995), it is known to reduce cytochrome C and to oxidise iron II and ascorbic acid (Halliwell and Gutteridge, 1989). The base like and reducing properties of the superoxide radical are increased when it is in an organic environment. In organic solution, it also acts like a nucleophile i.e. it is attracted to centres of positive charge. It can displace Cl from chloro compounds and also slowly oxidises \( \alpha \)-tocopherol to the tocopheryl radical as shown below.

\[
\begin{align*}
\text{Cl} & \quad \text{Cl} \\
\text{Cl} & \quad \text{Cl} \\
\text{Cl} & \quad \text{Cl} \\
\text{Cl} & \quad \text{Cl} \\
\text{C} & \quad \text{C} \\
\text{C} & \quad \text{C} \\
\text{O}_2 & \quad \text{O}_2^- \\
\text{Cl} & \quad \text{Cl} \\
\text{Cl} & \quad \text{Cl}
\end{align*}
\]

*Reaction with carbon tetrachloride to produce a peroxyl radical*
\[
\begin{align*}
O_2 + \text{tocopherol} \quad &\longrightarrow \quad \text{tocopherol ion} + HO_2^- \\
HO_2^- + \text{tocopherol} \quad &\longrightarrow \quad H_2O_2 + \text{tocopheryl radical} \\
O_2 + \text{tocopherol ion}^- \quad &\longrightarrow \quad O_2^- + \text{tocopheryl radical} \\
\end{align*}
\]

*Reactions with tocopherol to form the tocopheryl radical*

Biologically, the most important source of the superoxide radical is the electron transport chains of the mitochondria and endoplasmic reticulum. The oxidation of NADH and FADH₂ produced by the TCA cycle takes place via an electron transport chain that is situated in the inner mitochondrial membrane and is described in Fig. 15.2. NADH is oxidised to NAD⁺ by a multi-enzyme complex, NADH-coenzyme Q reductase and two electrons are passed to coenzyme Q. The acceptance of these electrons by coenzyme Q leads to the formation of partially reduced (semiquinone) and fully reduced quinones. The electrons are then passed via the coenzyme Q cytochrome c reductase enzyme complex to cytochrome c. Cytochrome c can accept one electron at a time by reducing FeIII to FeII. The multi-enzyme complex cytochrome c oxidase accepts electrons from cytochrome c, reoxidising the cytochrome c. One oxygen molecule is fully reduced to two molecules of water for every four electrons taken in by this oxidase complex. The energy released during this process is used to drive ATP synthesis. The cytochrome c oxidase enzyme complex keeps any radical intermediates of oxygen reduction tightly bound but other components of the ETC such as the NADH-coenzyme Q reductase complex and the reduced forms of coenzyme Q can leak electrons to oxygen which leads to the formation of the superoxide radical. This in turn can lead to an increase in hydrogen peroxide concentration through dismutation of the superoxide radical by
Fig 1.5.2 The Electron Transport Chain

\[
\begin{align*}
\text{NADH} & \quad 2e^- \quad \text{NADH-coenzyme Q reductase} \\
\text{NAD}^+ & \quad \text{E---FADH}_2 \\
& \quad \text{(protein-bound)} \\
& \quad \text{O}_2 \quad 2\text{H}_2\text{O} \quad \text{(per 4e}^-\text{taken in)} \\
\text{Cytochrome oxidase} & \quad \text{Cytochrome c redox cycle} \\
& \quad \text{CoQH}_2 \quad \text{cytochrome c reductase} \\
& \quad \text{CoQ}_10 \\
& \quad \text{Oxidized Q} \\
& \quad 1e^- \quad 1\text{H}^+ \\
& \quad \text{Partially reduced (semiquinone)} \\
& \quad 1e^- \quad 1\text{H}^+ \\
& \quad \text{Fully reduced (CoQH}_2) \\
\end{align*}
\]

Reproduced from Hallwell and Gutteridge (1989)
MnSOD that are located in the mitochondrial membrane. Hydrogen peroxide, if not removed, can form the hydroxyl radical via the Fenton reaction (see below). The hydroxyl radical is sufficiently reactive to abstract a hydrogen from a membrane lipid and initiate the lipid peroxidation chain reaction (see section 1.6).

A further source of free radicals is the cytochrome P450 superfamily of metabolising enzymes that are situated in large quantities in the endoplasmic reticulum of hepatocytes due to the xenobiotic detoxification function of the liver. The cytochrome P450 enzyme complex metabolizes xenobiotics using a mixed-function oxidase reaction that utilises molecular oxygen, adding one atom to the substrate and converting the other to water. The presence of a reducing agent is essential. The reaction is as follows:

\[ AH + O_2 + RH_2 \rightarrow A^\cdot OH + R + H_2O \]

where \( AH \) is the substrate and \( RH_2 \) is the reducing agent. Further metabolism may then occur (as described in section 1.1). In the liver, the electrons required for cytochrome P450 function are donated by NADPH via NADPH-cytochrome P450 reductase. This enzyme can leak electrons to oxygen, reducing it to form the superoxide radical, which, through formation of hydrogen peroxide and the hydroxyl radical, can initiate lipid peroxidation.

An additional source of superoxide in the liver may be the enzyme system desaturase that introduces carbon-carbon double bonds into fatty acids. Electrons are transported from NAD(P)H via a flavoprotein enzyme to cytochrome b_{5}. Cytochrome b_{5}
in turn passes the electrons to desaturase. Electrons may be leaked to oxygen from both cytochrome b₅ and the flavoprotein.

Further addition of an electron to oxygen leads to the formation of a peroxide ion. This is not a radical. With each addition of an electron the oxygen–oxygen bond is weakened and the addition of two or more electrons usually leads to the dissolution of the bond leaving two O₂²⁻ ions.

**Formation and reactions of the hydroxyl radical**

The spin restrictions inhibiting reaction of oxygen and non-radicals can be overcome during catalysis by transition metals. These metals, as well as having unpaired electrons, have variable valencies that make them very effective catalysts in oxidation/reduction reactions, explaining their presence at the active site of many enzymes. One very important reaction involved in radical production is the Fenton reaction, first observed by Fenton in 1894. This involves the reaction between an iron II salt and hydrogen peroxide which, via an intermediate complex, produces the hydroxyl radical, OH and is shown below:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{HO}^-
\]

OH⁻ is one of the most reactive species known and reacts with almost every type of molecule found in living cells. Due to its high reactivity, any hydroxyl radical formed will react at or very close to its site of formation, and although the radicals it forms are usually less reactive, most of the damage is done through further reactions of these.
radicals. The damage seems to affect DNA in particular, causing single-strand breaks and DNA-protein cross links (Halliwell and Gutteridge, 1989). The formation of these radicals is shown in Fig. 15.3.

Other radicals found in biological systems include thyl, carbon centred, nitrogen centred and phosphorous centred radicals.
Fig 15.3 The Formation of Reactive Oxygen Species

Reproduced from Singal et al (1988)
Biological functions of free radicals

Free radicals are also produced by the body for specific purposes. The superoxide radical is generated in small amounts by lymphocytes and fibroblasts, possibly as a growth regulator (Rice-Evans, 1995). It is also used as part of the defense mechanism of phagocytes where it is firstly converted to hydrogen peroxide which is used to kill engulfed bacteria.

The role of xenobiotic metabolism in the occurrence of oxidative stress

The biotransformation function of the liver as described in section 1.1, ensures that the liver is a prime site for the production of free radicals and the occurrence of oxidative stress. Oxidative stress, defined as a disturbance in the pro-oxidant – antioxidant balance of a cell can occur, for example, when an excess of chain reaction initiating radicals are formed. During the metabolism of halogenated hydrocarbons such as carbon tetrachloride, the abstraction of the halogen by the haem group of the cytochrome P450 enzymes leads to the formation of carbon centered radicals that are capable of initiating such chain reactions. Xenobiotics such as quinones can become involved in cycles of oxidation-reduction reactions. Cytochrome P450 reductase catalyses the reduction of quinones by nicotinamide nucleotides. The resulting semiquinone can be either detoxified by reaction with a nucleophile such as glutathione or it can undergo a one-electron oxidation back to the original quinone but with the concurrent production of the superoxide anion. The quinone can again be reduced to the semiquinone commencing a cycle of reactions with constant production of the superoxide anion and constant reduction in the concentration of the nicotinamide nucleotides (Castell et al, 1997). Other
xenobiotics which can undergo such cycles of reactions are catechols, pyridinium
derivatives, N-nitrosureas and aromatic nitro and nitroso compounds. A diagrammatic
representation of this redox cycling is shown in Fig. 154

Xenobiotic metabolism can also be responsible for a reduction in cellular glutathione
levels. The role glutathione plays in alleviating oxidative stress is described in section
154. During metabolism glutathione may be conjugated to metabolites. These metabolites
are further processed in other tissues such as the kidney but the glutathione, now in the
form of mercapturic acids is excreted in the urine and therefore lost. During functioning
of the enzyme glutathione peroxidase, glutathione is oxidised. The reduced form of
glutathione can be recovered via the action of glutathione reductase. However, if this
enzyme is overloaded, the concentration of cellular oxidised glutathione increases and the
oxidised glutathione is removed from the cell due to its toxic effects. This again leads to
loss of cellular glutathione (Castell et al., 1997)

16 Lipid Peroxidation

One of the effects of radicals in biological systems is to induce lipid peroxidation.
Lipid peroxidation can be described as the oxidative deterioration of polyunsaturated
lipids, a polyunsaturated lipid being a lipid with more than two carbon-carbon covalent
double bonds. Such polyunsaturated fatty acids (PUFA) are present in membranes where
they reduce the membrane melting point so that it has the chemical nature and viscosity
of a light oil. Damage to these PUFAs results in the loss of membrane fluidity. Most
membrane lipids are amphipathic; i.e., they have polar and non-polar groups. They are
usually long hydrophobic fatty acid side chains joined by ester bonds to glycerol. Animal
Fig. 1.5.4 Xenobiotic Redox Cycling

\[
\begin{align*}
\text{Glycolysis/Krebs Cycle} & \quad \text{Pentose Cycle/Lipid oxidation} \\
\text{O}_2 \quad \text{Quinone} & \quad \text{NADH} \\
\text{O}_2 \quad \text{Semi-quinone} & \quad \text{NADPH} \\
\text{SOD} & \quad \text{NADP}^+ \quad \text{GSSG} \\
\text{Catalase} & \quad \text{H}_2\text{O}_2 \quad \text{M}^+ \quad \text{OH}^- \quad \text{OH} \\
\text{GSH} & \quad \text{GSH Peroxidase} \quad \text{GSSG} \\
\downarrow & \quad \text{GSH} \quad \text{GSSG} \\
\text{2H}_2\text{O} & \quad \text{ROOH} \quad \text{GSSG} \\
\text{GSSG} & \quad \text{ROH} \quad \text{GSH}
\end{align*}
\]

Reproduced from Castell et al (1997)
cell lipids usually have unbranched side chains composed of even numbers of carbon atoms, generally between 14 and 24 in length. Any double bonds are in the cis configuration introducing "kinks" into the side chain. The phospholipids are the most dominant lipids found in animal cells, although there is much sphingolipid and cholesterol in some cells, especially in plasma. The membranes of organelles rarely have much sphingolipid or cholesterol (Halliwell and Gutteridge, 1989).

The first step in lipid peroxidation (initiation) requires a species that is sufficiently reactive to remove a hydrogen atom from a methylene group in a fatty acid. The presence of a double bond in the fatty acid weakens the carbon-hydrogen bonds on either side of it, making the removal of a hydrogen atom easier. The hydroxyl radical is reactive enough to carry out this process while the superoxide radical is not. The protonated form of the superoxide radical, the hydroperoxyl radical has been shown to abstract a hydrogen atom from some fatty acids in vitro but has not yet been proved to initiate peroxidation in cell membranes (Halliwell and Gutteridge, 1989). Once the hydrogen atom has been removed from the fatty acid, the fatty acid may form a conjugated diene to become more stable but under aerobic conditions it is more likely to combine with oxygen to form a peroxyl radical. These peroxyl radicals are capable of abstracting hydrogen atoms from a different lipid molecule, so propagating the chain reaction. The peroxyl radical can combine with the hydrogen it abstracts to form a lipid hydroperoxide, it can also form cyclic peroxides (Singal et al, 1988). These products do not participate in the chain reaction any further and so this is the termination of the chain reaction process. The presence of iron has been found to stimulate lipid peroxidation. As iron is involved in the production of hydroxyl radicals via the Fenton reaction, this is to be expected. However,
Iron-stimulated lipid peroxidation has not been found to be inhibited by either catalase or hydroxyl radical scavengers (Tyler, 1975; Halliwell and Gutteridge, 1989). A theory has been proposed, therefore, that initiation of iron-stimulated lipid peroxidation is brought about by a species other than the hydroxyl radical, possibly a ferryl or perferryl species. The presence of iron can also bring about the decomposition of lipid peroxides to form alkoxy and peroxy radicals. These radicals can abstract hydrogen atoms and initiate further lipid peroxidation (Marnett and Wilcox, 1995). The end-products of metal-stimulated lipid peroxidation include epoxides, carbonyl compounds and hydrocarbon gases. This reaction is shown in Fig. 16.1.

The effects of lipid peroxidation include the cross-linking of membrane proteins, the inhibition of protein synthesis, the inactivation of surface receptors and the inhibition of enzymes that contain NH$_2$ or SH groups. It is also likely that cholesterol becomes oxidised. These effects all lead to an overall decrease in membrane fluidity and an increase in membrane "leakiness" i.e., substances can cross the membrane which normally would not be able to. There may be an eventual complete loss of membrane integrity.

1.7 Antioxidant Defense

As can be seen from section 1.5 above, the body is exposed to many harmful radicals both from the environment and through in vivo production. There exists, therefore, a system to protect against the harmful effects of these radicals and to decrease the incidence of lipid peroxidation. The antioxidant defense system is composed of a group of antioxidant enzymes and a battery of low molecular weight compounds with
Fig. 1.6 Lipid Peroxidation

Fatty acid with 3 double bonds

\[
\begin{align*}
\text{Hydrogen abstraction} & \\
\text{Molecular rearrangement} & \\
\text{Conjugated diene with UV absorbance at 234 nm} & \\
\text{Oxygen uptake} & \\
\text{Peroxy radical abstracts} \\
\text{H}^* \text{ from another fatty acid causing an autocatalytic chain reaction} & \\
\text{Lipid hydroperoxide} & \\
\text{Cyclic peroxide} & \\
\text{Cyclic endoperoxide} & \\
\text{Fragmentation to aldehydes (including malondialdehyde) & polymerization products} & 
\end{align*}
\]

Reproduced from Halliwell and Gutteridge (1989)
antioxidant function The antioxidant enzymes are catalase, glutathione peroxidase (GPx), glutathione reductase and superoxide dismutase. The cellular locations of the antioxidant defense system are shown in Fig 17.1

Antioxidant Enzymes

Catalase Catalase catalyses the conversion of hydrogen peroxide to water and oxygen as shown

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The oxygen produced in this reaction is in the ground state, effectively stopping any further radical activity. In animals, it is found in all organs, especially the liver and erythrocytes. It is composed of four protein subunits. Each subunit contains a haem group bound to the active site and usually one bound NADPH molecule. The mechanism of reaction is thought to be as follows

Catalase – Fe(III) + H_2O_2 \rightarrow compound I

Compound I + H_2O_2 \rightarrow catalase – Fe(III) + H_2O + O_2

The structure of compound I is not known as the extensive delocalisation of the charge over the haem rings makes it very difficult to determine. Catalase has a very high affinity for the destruction of hydrogen peroxide and in the presence of a steady supply of
Fig 17.1 Cellular antioxidant defense

Reproduced from Machlin and Bendich (1987)
hydrogen peroxide, can also have some peroxidase-like activity due to the action of compound I. Compound I can oxidise ethanol, methanol, acetaldehyde, nitrous oxide and possibly elemental mercury. If the catalase subunits are separated, they have a wider range of peroxidase activity. Catalase activity is mainly located in the peroxisome (Halliwell and Gutteridge, 1989). Catalase can, however, be inactivated by hydrogen peroxide due to the formation of a catalase-hydrogen peroxide complex which is stable in the presence of excessive amounts of hydrogen peroxide and at low pH (Singh et al., 1993).

**Glutathione Peroxidase** Glutathione peroxidase removes hydroperoxide and organic peroxides by catalysing a reaction between them and reduced glutathione to form oxidised glutathione and water as shown:

\[
\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O}
\]

It is specific for glutathione although not for hydrogen peroxide and has a high activity in liver tissue. It is composed of four protein subunits with a selenium atom at the active site. The glutathione apparently reduces the selenium and then the reduced form of the enzyme reacts with the hydrogen peroxide. GPx is mainly situated in the cytosol and the mitochondrial matrix (Halliwell and Gutteridge, 1989). The structures of reduced and oxidised glutathione are shown in Fig 17.2.
Fig 172 The structure of reduced and oxidised glutathione

\[
\begin{align*}
\text{GSH} & : \quad \text{Gly} - \text{Gly} - \text{Gly} - \text{Cys} - S - S - \text{Cys} - \text{Glu} - \text{Glu} \\
\text{GSSG} & : \quad \text{Gly} - \text{Gly} - \text{Gly} - \text{Cys} - S - S - \text{Cys} - \text{Glu} - \text{Glu}
\end{align*}
\]
Glutathione reductase reduces the oxidised glutathione produced by GPx ensuring a steady concentration of reduced glutathione in the cell. The reaction is as follows:

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Glutathione reductase can also catalyse the reduction of mixed disulphides. It is composed of two protein subunits with FAD at the active site. NADPH reduces the FAD which passes electrons onto a disulphide bridge between two cysteine residues in the protein. The two thiol groups formed interact with the oxidised glutathione reforming the protein disulphide. Some animal tissues have a non-selenium GPx which acts on organic peroxides but not on hydrogen peroxide. It is located in the cytosol with small amounts in the mitochondria (Halliwell and Gutteridge, 1989).

**Superoxide dismutase** Two types of superoxide dismutase have been found in animal cells. These are the copper-zinc superoxide dismutase (CuZnSOD) and the manganese superoxide dismutase (MnSOD). CuZnSOD is very stable. It is resistant to heating, proteases and denaturation by SDS or urea, but is inhibited by cyanide. It is found in nearly all eukaryotic cells and is situated in the cytosol, with a small amount present in the lysosome, the mitochondrial matrix and possibly some in the nucleus. It has a molecular mass of approximately 32000 and contains two protein subunits. Each subunit has an active site containing a Cu\(^{2+}\) and Zn\(^{2+}\) ion. While the copper ion is involved in the catalytic process, the zinc ion is not. It seems to be present to stabilise the enzyme. CuZnSOD catalyses the dismutation of the superoxide radical to form hydrogen peroxide.
and oxygen. The copper ions seem to function in the dismutation reaction by undergoing alternate oxidation and reduction:

\[
\text{Enz} - \text{Cu}^{2+} + \text{O}_2 \rightarrow \text{Enz} - \text{Cu}^+ + \text{O}_2 \\
\text{Enz} - \text{Cu}^+ + \text{O}_2 + 2\text{H}^+ \rightarrow \text{Enz} - \text{Cu}^{2+} + \text{H}_2\text{O}_2
\]

MnSOD is a less stable enzyme than CuZnSOD. It is also heat labile and the activity decreases at alkaline pH. It is, however, insensitive to cyanide. MnSOD comprises 10% of total SOD in rat liver, while the percentage in human liver is higher (Halliwell and Gutteridge, 1989). It is situated in the mitochondrial matrix, although in human liver there is some MnSOD situated outside the mitochondria. In higher organisms, MnSOD is composed of four protein subunits with 0.5 to 1 manganese ion per subunit. The amino acid sequence of MnSOD is highly conserved and it is unrelated to CuZnSOD. MnSOD catalyses the same overall reaction as CuZnSOD i.e.

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

but the mechanism is different. The manganese ion undergoes changes in its valency state but the exact nature of this change is not known. It is not a simple two stage mechanism (Halliwell and Gutteridge, 1989).

The introduction of SOD into mammalian cells has led to resistance to hyperoxia and to paraquat and yeast with a SOD defect was more susceptible to damage by dioxygen, paraquat and irradiation (Halliwell and Gutteridge, 1989). This protective...
effect indicates that the superoxide radical is toxic although it is not a very reactive radical. The toxic effects could be due to the production of the hydroxyl radical from superoxide. Secondly, both catalase and GPx are inactivated by the presence of the superoxide radical. This would leave cells open to the damage caused by hydrogen and organic peroxides. SOD protects against the inactivation of these enzymes (Fridovich, 1986).

The amino acid sequence of CuZnSOD is highly conserved, the only known mutations being associated with Amyotrophic Lateral Sclerosis (ALS), also known as Charcot disease. This disease involves the late onset of a progressive neurodegenerative disorder affecting the α-motor neurons and upper motor neurons of the pyramidal tract (Luche et al., 1997).

**Antioxidant Molecules**

The low molecular weight molecules involved in antioxidant defense are formed both *in vivo* and ingested in the diet. Halliwell (1995) defines an antioxidant molecule as “any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.” Antioxidants may be primary i.e., they are preventative. They inhibit oxidation by reducing the rate of initiation of free radical chains. Usually they convert the products of prior oxidation, which may initiate further oxidation, to harmless substances. Secondary antioxidants are chain-breaking antioxidants; they trap the chain-propagating radicals so reducing the length of the chain (Burton et al., 1983). Antioxidant molecules include ascorbic acid, glutathione, uric acid, metallothioneins, β-carotene, and α-tocopherol.
Ascorbic acid, or vitamin C, is a water-soluble antioxidant and is mostly found in muscle, the adrenals and the eye. Its antioxidant properties lie in its ability to be easily oxidized, either by one or two electron transfer. Both the 2- and 3- hydroxyl groups (structure shown in Fig 1.7.3) must be unsubstituted to ensure antioxidant activity (Rice-Evans, 1995). Antioxidant activity involves a two-electron oxidation to form dehydroascorbate with the intermediate formation of an ascorbyl radical. Dehydroascorbate is unstable and may be reduced back to ascorbate or hydrolysed to diketogluconic acid. Injection of dehydroascorbate into animals was found to affect insulin secretion and induce diabetes (Halliwell and Gutteridge, 1989). Ascorbic acid may detoxify organic radicals. It scavenges singlet oxygen and reduces thyl radicals. It can regenerate vitamin E by reducing α tocopheryl radicals at the surface of membranes but may provoke the formation of free radicals when in the presence of transition metals (Machlin and Bendich, 1987). It can reduce Fe(III) to Fe(II) and in the presence of hydrogen peroxide may stimulate the Fenton reaction. However, it can also scavenge hydroxyl radicals so the overall effect of ascorbic acid depends on its concentration (Halliwell and Gutteridge, 1989). A reduced ascorbic acid concentration is found in patients with sickle-cell anemia, rheumatoid arthritis, adult respiratory disease syndrome and iron overload disease. A symptom of all of these diseases is an increase in available metal ions and the low ascorbic acid concentration may be a defensive response to decrease the occurrence of the Fenton reaction (Halliwell and Gutteridge, 1989).
Ghosh *et al* (1997) studied lipid peroxidation in guinea pig microsomes due to NADPH-initiated cytochrome P450 activity, in the absence of free iron. After a 30 minute period the conjugated dienes and lipid hydroperoxide produced were measured. A time-dependent increase in both these parameters was observed. Co-incubation with ascorbic acid completely prevented this increase. MDA was not produced in this study as the breakdown of lipid hydroperoxide to MDA is iron catalyzed. The lipid hydroperoxide was isolated and incubated with ADP-Fe³⁺ to produce MDA. In this case ascorbic acid was found to increase MDA levels demonstrating the pro-oxidant effect of ascorbic acid in the presence of iron. The effects of SOD (100 units), catalase (40 μg), glutathione (100 μM), α-tocopherol (20 μM), mannotol (20 mM), histidine (10 mM), uric acid (10 mM), thiourea (10 mM), β-carotene (20 μM) and probucol (20 μM) were studied and were found to yield conjugated dienes, lipid hydroperoxide and MDA levels similar to those in
the absence of any antioxidant i.e. they had no antioxidant effect in this situation. The effectiveness of ascorbic acid and ineffectiveness of the other known antioxidants in preventing lipid peroxidation is thought to be due to the manner in which lipid peroxidation was induced. The authors proposed that the perferryl moiety P450 Fe³⁺O₂⁻ initiated lipid peroxidation by abstracting a methylene hydrogen from polyunsaturated lipid to form lipid radical which produced peroxyl radicals when combined with oxygen and this peroxyl radical propagated the peroxidation chain reaction. The authors concluded that ascorbic acid specifically prevented initiation of lipid peroxidation by interacting with P450 Fe³⁺O₂⁻.

**Uric Acid**

Uric acid is a powerful scavenger of singlet oxygen, peroxyl radicals and hydroxyl radicals. It is produced as an endproduct of purine metabolism. During scavenging of hydroxyl radicals, urate peroxyl radicals are produced which, although less reactive than hydroxyl radicals, may inactivate enzymes. These peroxyl radicals may be reduced by ascorbic acid. The reaction between uric acid and the hydroxyl radical is shown in Fig 1.7.4.

**Fig 1.7.4 The reaction between uric acid and the hydroxyl radical**

\[
\begin{align*}
R\cdots\text{C} & \cdots\text{H} + \text{OH} \rightarrow R\cdots\text{C} \cdots\text{H}_2\text{O} \\
R\cdots\text{C} & \cdots\text{H}_2\text{O} + \text{O}_2 \rightarrow R\cdots\text{CO}_2
\end{align*}
\]

*Urate Peroxyl radical*
**Metallothioneins**  Metallothioneins are proteins with a molecular weight of approximately 6500. They are found in the cytosol of eukaryotic cells especially the liver, kidney and gut. They are sulphur rich and can bind and store metal ions. Binding is achieved by association of the cysteine -SH groups with the metal ions. For example, cadmium and zinc are linked to four cysteine thiolate ligands in a tetrahedral arrangement (Halliwell and Gutteridge, 1989). They can scavenge singlet oxygen and hydroxyl radicals. The removal of copper ions may decrease some radical generation and the release of zinc may decrease lipid peroxidation.

**Glutathione**  Reduced glutathione, as seen previously, is the substrate for GPx activity. It can also scavenge singlet oxygen and hydroxyl radicals. It can reactivate some enzymes that have been inactivated by high oxygen concentrations and is also a cofactor for some enzymes. It is usually present in high concentrations in cells. A high concentration of hydrogen peroxide or hydroxyl radical can lead to a change in the normal reduced / oxidised glutathione ratio and the concentration of oxidised glutathione may increase. Oxidised glutathione inactivates some enzymes such as phosphorylase phosphatase and adenylate cyclase and also inhibits protein synthesis. The liver releases oxidised glutathione when it is under oxidant stress (Halliwell and Gutteridge, 1989).

**β Carotene**  β Carotene is the most effective quencher of singlet oxygen known in nature. It is a precursor of vitamin A although vitamin A itself has no antioxidant activities. It has been found in both membranes and serum (Machlin and Bendich, 1987). β Carotene is a naturally occurring pigment and a high level of β carotene has been...
linked with a low level of the incidence of myocardial infarction. It has been found to act synergistically with α tocopherol in the inhibition of lipid peroxidation in rat liver microsome extracts (Bonorden and Pariza, 1994) When functioning as a chain-breaking antioxidant, β carotene brings about a shift in the oxidation equilibrium toward polymer formation and away from hydroperoxide formation. This means that there is a reduction in the incidence of free radical hydroperoxide decomposition products (Bonorden and Pariza, 1994). The structure of β carotene is shown in Fig 17.5.

**Fig 17.5 The structure of β carotene**

The Physician's Health Study which was commenced in 1982 involved the study of the health benefits of dietary β carotene. Administration of 50 mg β carotene on alternate days resulted in a 44% reduction in major coronary events i.e. myocardial infarction, revascularisation and death (Rice-Evans, 1995). However, a study in Finland of elderly male long term smokers found an increase in the risk of lung cancer when the diet was supplemented with 20 mg of β carotene a day (α-Tocopherol, β-Carotene Prevention Study Group, 1994)
\( \alpha \text{Tocopherol} \) \( \alpha \) Tocopherol, also known as vitamin E although vitamin E is really the collective name for a group of tocopherols - \( \alpha, \beta, \gamma, \delta \) of which \( \alpha \) tocopherol is the most biologically active, is found in the lipid fraction of living organisms, the highest concentrations being found in retinal rods, lipoproteins and the adrenal glands. The main dietary source is vegetable oil, but it is also found in liver, eggs, cereals and legumes. Vitamin E is highly hydrophobic and requires adequate concentrations of bile salts for uptake. It is transported through the blood in low density lipoproteins (LDLs) and released when these LDLs are taken up into cells. It is presumed to be localised in cells in association with the intracellular membrane. It is possible that there is an interaction between the tocopherol side chain and the arachidonyl residues in membrane phospholipids (Diplock, 1983). \( \alpha \) Tocopherol functions as a chain-breaking antioxidant by trapping peroxyl radicals. It has a phenolic hydroxyl group and can transfer a hydrogen to the free radicals (Vasavi \textit{et al}, 1994) as shown:

\[
\begin{align*}
\text{ROO} + \text{AH} & \rightarrow \text{ROOH} + \text{A} & \text{reaction 1} \\
\text{ROO} + \text{A} & \rightarrow \text{ROOA} & \text{reaction 2}
\end{align*}
\]

The effectiveness of an antioxidant depends on its reactivity towards the peroxyl radical, i.e., reaction 1 is rate limiting and the higher the rate constant for this reaction, the more effective the antioxidant.
With relation to α tocopherol, the second ring holds the oxygen so that the p-type lone pair makes an angle of approximately 74° with the aromatic ring. The orbital of the lone pair can overlap the orbital containing the unpaired electron and so stabilize the phenoxy radical. The stability of this radical means that the bond between the O-H in the phenol is weak making it readily susceptible to cleavage by an attacking peroxyl radical (Burton et al., 1983). One tocopherol molecule can stop two different chains as the tocopheryl radical is too weak to initiate a chain reaction itself. This is due to the fact that the unpaired electrons can be delocalized into the aromatic ring structure increasing the stability of the molecule (Halliwell and Gutteridge, 1989).

The principle function of α tocopherol is to protect the lipid material of an organism from spontaneous autoxidation. It can react directly with peroxyl, hydroxyl and superoxide radicals (Burton et al., 1983). Vitamin E supplementation (400 mg/kg) has been shown to reduce the number of chemically-induced tumors in rats. It has also been
found to inhibit the conversion of nitrates to nitrosamine. This is an important step in cancer causation (Vasavi et al., 1994). However, Mak et al. (1996) found that α tocopherol supplementation – 0.3 to 3 mmol per kg had no effect on the lipid peroxidation or the hepatocellular damage caused by carbon tetrachloride in female Balb/c mice. In the same study, human erythocyte membrane lipids were prepared and exposed to different concentrations of α tocopherol. At a concentration of 1 mM α tocopherol was found to increase TBARS production, in comparison with control, over an initial 20 min period but then suppress any further TBARS production up to 120 minutes, meaning that the final TBARS concentration was significantly (p<0.05) less than control. The lipid peroxidation in this study was induced with iron catalysis and the ratio of Fe^{3+}/Fe^{2+} is important. Mak proposed that α tocopherol was able to induce the conversion of Fe^{3+} to Fe^{2+} so that lipid peroxidation occurred more quickly than under control conditions but that once lipid peroxidation had been initiated, α tocopherol functioned as an antioxidant and so decreased overall lipid peroxidation. α Tocopherol has been found to return levels of catalase and superoxide dismutase to control and below control levels in chicken embryo fibroblasts which have been stressed with paraquat (Lawlor and O’Brien, 1994).

Severe prolonged vitamin E deficiency, as is seen in the disease abetalipoproteinaemia where absence of the synthesis of apolipoprotein B and therefore chylomicrons means that it is not possible to transport dietary lipid from the intestinal mucosal cells, leads to the eventual onset of neuropathy, retinal degradation and abnormally shaped erythrocytes (Halliwell and Gutteridge, 1989).

A cross-cultural epidemiological study carried out by the World Health Organisation showed an inverse correlation between plasma vitamin E levels and
mortality from ischaemic heart disease in middle aged men from 16 different population groups (Rice-Evans, 1995) The anti-cancer effect of natural antioxidants was studied in Linxian, a rural area in northern China where the death rate due to cancer of the stomach and oesophagus is very high, ten times that of the USA. Supplementation of the diet with a mixture of vitamin E, β carotene and selenium significantly reduced the rates of death due to individual cancers (between 13 and 21%) and also reduced the overall mortality rate by 9% (Rice-Evans, 1995).

Antioxidants are also used to preserve foodstuffs during processing and storage. Huber et al. (1995) demonstrated the efficacy of a tocopherol blend in reducing the production of some cholesterol oxides during processing and storage of egg yolk. The tocopherol blend, Tenox GT-2 (contained 15-20% α, 15-20% γ and 60-65% δ tocopherols), reduced the levels of 7-ketocholesterol, 7-α and 7-β hydroxycholesterol following spray drying and accelerated storage. The levels of cholestanetriol and cholesterol 5,6-epoxide were not affected by the presence of the tocopherol blend.

**Antioxidant interaction**

There are many interactions between the different antioxidant micronutrients that lead to more effective antioxidant protection. Vitamin C regenerates the reduced tocopherol from the tocopheroxyl radical. Vitamin E protects the conjugated double bonds of β carotene from oxidation. Vitamin E can also protect against many of the symptoms of selenium deficiency and vice versa (Machlin and Bendich, 1987). These interactions are shown diagrammatically in Fig. 17.7.
Fig 17.7 Antioxidant interaction

Reproduced from Machlin and Bendich (1987)
Conjugated Linoleic Acid

Conjugated linoleic acid, a mixture of isomers of conjugated dienoic derivatives of linoleic acid, is being extensively researched due to its purported antioxidant and anticarcinogenic properties. Ha et al. (1990) demonstrated antioxidant effects of CLA after observing that CLA reduced the incidence of tumors in mice exposed to benzo(a)pyrene to induce fore-stomach neoplasia. Linoleic acid (375 μmol) was incubated in aqueous ethanol for 15 days at 40°C with 375 nmol CLA, ascorbic acid, BHT or α tocopherol. The extent of oxidation was then determined by adding ferrous ammonium sulphate and thiocyanate and measuring the absorbance at 480 nm. CLA was found to be more effective at inhibiting oxidation of linoleic acid than ascorbic acid and α tocopherol and to be almost as effective as BHT. CLA was also found to inhibit singlet oxygen-catalyzed linoleic acid hydroperoxide formation in aqueous ethanol by 20% at a ratio of 1:10 parts CLA to linoleic acid (Bonorden and Pariza, 1994). CLA is less efficient than β carotene at quenching singlet oxygen, but more efficient than α tocopherol in a chloroform/ethanol solvent system (Bonorden and Pariza, 1994). Ip et al. (1991) fed female Sprague Dawley rats 0 to 1.5% CLA for two weeks before exposing them to dimethylbenz(a)anthracene to induce mammary tumors. The feeding was continued for a further 24 weeks while the tumors developed. CLA was found to decrease size and yield of tumors up to a concentration of 1%. No further benefit was found in increasing the concentration of CLA to 1.5%. In the same study lipid peroxidation was assessed by quantifying the TBARS present in both liver and mammary tissue. CLA was found to have no effect on the TBARS concentration in liver tissue although a significant,
but not dose responsive, decrease in mammary tissue was found. The study also included animals who had been fed 0.05% vitamin E or 0.1% BHA. A significant (p<0.05) decrease in TBARS was found in both liver and mammary tissue of these animals (Ip et al., 1991).

A definite mechanism for the proposed antioxidant functions of CLA has not been agreed. The conjugated system (see Fig 1 7 8) is very susceptible to free radical attack, indicating that CLA may trap chain-propagating lipid radicals. This may result in a shift in the reaction equilibrium towards termination, a similar effect to that of β carotene. Ha et al. (1990) after observing a change in the UV absorption profile of CLA when it was incubated with linoleic acid, suggested that a β-hydroxy acrolein moiety within the CLA molecule would explain the appearance of a UV maximum at 268 nm, which they observed. They suggested that such a structure could arise from reaction of CLA with peroxyl or hydroxyl radicals, followed by molecular oxygen. The antioxidant activity of such a structure was suggested to result from one of two possibilities. Firstly, resonance enolization of the β-hydroxy acrolein moiety may occur or this structure may chelate iron, so inhibiting the Fenton reaction (Ha et al., 1990).

**Fig 1 7 8 The structure of the cis9, trans11 isomer of CLA**
The fact that Ha's research (1990) into the antioxidant properties of CLA had been carried out in a phosphate buffer ethanol mixture, which does not reflect physiological conditions and that Ip's work (1991) yielded evidence that CLA's antioxidant activity was specific to mammary tissue and that a lower concentration of vitamin E and BHA had more pronounced antioxidant effects led to further studies using defined model systems and more precise analytical methodology. Phospholipid membranes composed of 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) were prepared and subjected to metal ion and metal ion-independent stress (the oxidants used were hydrogen peroxide in the presence of ferrous ammonium sulphate for metal ion and 2,2'-azobis(2,4-dimethylvaleronitrile) and 2,2'-azobis(2-amidinopropane) hydrochloride for metal ion independent stress) at various time points the membranes were analysed by GC-MS for residual non-oxidised fatty acids. By analysing for the fatty acids that had not yet been oxidised rather than for any one particular end-product, the major inaccuracy in lipid peroxidation determination had been removed. 0.75 μM α-tocopherol and 0.75 μM BHT were found to be excellent radical scavenging antioxidants whereas CLA was not found to be at all effective in that respect (Van den Berg et al., 1995). These experiments were carried out using metal ion independent stress. Metal ion dependent oxidative stress was used to test the theory that CLA may act as a chelator and so reduce the metal ions available for the Fenton reaction. Incorporation of vitamin E (1 μM) into the PLPC membranes led to a temporary suppression of oxidation when the membranes were exposed to the hydrogen peroxide/ferrous ammonium sulphate system and 50 μM EGTA was found to completely prevent oxidation. CLA was found to have no significant effect on the oxidation of PLPC membranes. This same result was obtained when
experimental conditions were altered and concentrations of 1-10 μM CLA were used.

Van den Berg therefore states that CLA does not seem to act as an antioxidant at all. This, however, does not explain the decrease in TBARS observed in mammary tissue of rats fed 1% CLA (Ip et al., 1991) and demonstrates the necessity of further research into the possible antioxidative properties of CLA.
CHAPTER 2

Validation of a Liver Model System for Short – Term Biochemical Studies
2.1 Introduction

Liver cells are now routinely prepared by perfusing the liver and disrupting the intracellular matrix with collagenase. Before the use of collagenase in 1967 by Howard et al or the introduction of perfusion in 1969, by Berry and Friend non-enzymatic methods were employed for the isolation of hepatocytes. These methods were invariably harsh and produced damaged cells restricting their use. It was necessary to employ a mechanical method to separate the cells from the matrix. Such methods included homogenisation, forcing the tissue through screens of stainless steel, silk or cheesecloth, pipetting, and shaking with glass beads (Seglen, 1976). The severity of these methods left cells damaged or dead. Although mitochondrial respiration was observed in the preparations, this can occur in non-intact cells and was only seen when the cells were incubated in medium capable of sustaining isolated mitochondrial suspensions (Berry et al, 1991).

Liver perfusion involves cannulation of the portal vein and, if recirculation is being used, of the caval vein also. The perfusate is introduced via the portal vein and so flushes blood out of the liver. The enzyme collagenase is included in one of the perfusion solutions. This acts on collagen whose removal detaches the cells from the tissue. During perfusion, the liver swells, and after perfusion, the liver has a blanched appearance. Removal of calcium via preperfusion with a chelator such as EGTA has been found to enhance separation of the cells (Seglen, 1976). Ca$^{2+}$ is involved in cellular adhesion, and its removal brings about irreversible changes thought to be caused by the removal of a Ca$^{2+}$-dependent adhesion factor. This adhesion factor possibly makes up the core of the desmosomes (one of the junctional complexes between cells) and due to its removal, the hemidesmosomes move apart. Collagenase, however, requires Ca$^{2+}$ which enhances its
activity. The two-step perfusion procedure was developed to address this problem
(Seglen, 1976). Initially, the liver is perfused with a Ca\(^{2+}\) free solution that contains a
chelating agent such as EGTA or EDTA. The removal of this ion initiates the breakdown
of the extracellular material. A solution containing both Ca\(^{2+}\) and collagenase is then
employed, which completes the dispersion of the cells. The collagenase generally used is
extracted from *Clostridium histolyticum* and is used in crude form. This collagenase
dissolves incompletely in buffer and retains much of its proteolytic activity. Collagenase
activity in the liver is optimal at a pH of 7.5. In order to maintain this pH, all solutions
used are at a pH of 7.5 and, as the liver acidifies the perfusing solutions, a buffering
system is used. Krebs-Henseleit bicarbonate saline has been used as the perfusing
medium as it has a high buffering capacity when used in an atmosphere containing 5%
CO\(_2\). The inclusion of HEPES, which does not require CO\(_2\), has also been found effective
as a buffering system (Berry et al, 1991).

Oxygenation during perfusion is often considered essential. This is to avoid the
degradation of ATP to inosine and to conserve gluconeogenesis. For long periods of
perfusion, oxygenation is very important but for short term – up to 30 minutes, it does not
seem necessary. Berry et al (1991) showed that hepatocytes prepared without
oxygenation showed no evidence of cellular detenoration, nor diminution of ATP or in
 gluconeogenesis. On occasion, however, cells prepared in this manner did have a high
percentage of damaged cells with considerable membrane blebbing due to hypoxia
indicating a possible case for oxygenation even for short-term perfusions (Berry et al,
An optimal flow rate must be used as problems are associated with either too high or too low a flow rate. Due to the low pressure created when a low flow rate such as 5 ml/minute is used, a uniform perfusion is difficult to achieve. The inclusion of erythrocytes or albumin in the perfusion media may benefit the process by increasing the viscosity and so increasing the pressure. If the flow rate is too high, disruption of cells may occur leading to the preparation of a high quantity of damaged cells. A flow rate of 50 ml/minute has been used by Seglen (1976). With such flow rates, however, it is essential to use recirculation to conserve reagents.

Isolated hepatocytes have been used to study the biotransformation of both endogenous compounds and exogenous drugs and chemicals. The high concentration of biotransformation enzymes present in the hepatocyte make this the cell type of choice for both the study of the xenobiotics biotransformed and of the biotransformation enzymes themselves (Blaauboer et al., 1994). This biotransformation function leads to a role for isolated hepatocytes in the determination of the possible cytotoxicity of the metabolites produced by biotransformation and also of the parent compounds. Some compounds become genotoxic following bioactivation and the use of hepatocytes as a test system to identify such genotoxins is a recognised protocol for mutagenicity testing (Blaauboer et al., 1994). The use of hepatocytes from different species facilitate inter-species comparisons with respect to biotransformation and toxicity of chemicals (Blaauboer et al., 1994).

Unfortunately, there is no standardised methodology for the isolation of hepatocytes, making correlation between different laboratories difficult. In 1975, Edwards published methodology for the isolation of rat hepatocytes involving deep
anaesthesia of the rat using sodium pentobarbital. Initial perfusion was carried out using a
calcium-free solution of Hanks' buffer, pH 7.4, followed by perfusion with bicarbonate-
free Swim's S-77 to which 20 mg collagenase was added (Edwards, 1975). Ingebritsen et
al (1979) used Krebs' bicarbonate buffer to isolate hepatocytes from Wistar rats which
were sacrificed prior to cell isolation. Princen and Meijer (1990) included a trypsin
inhibitor in the perfusion medium and O'Malley et al (1981) used 80 mg hyaluronidase
as well as 50 mg collagenase to achieve cell separation. The Hepatocyte User Group
(HUG) conducted a survey in 1993 to assess the level of variation in methodology used
for hepatocyte isolation. There were twenty-nine respondents, working with hepatocytes
from rat, mouse, sheep, monkey, pig, chicken, cow, dog, guinea pig, gerbil, goat, hamster
and humans. 32% stated that the animal was sacrificed prior to cell isolation, the methods
used being cervical dislocation, asphyxiation with CO₂ or ether, overdose of barbiturate
or the harvesting of material from a biopsy or abattoir. The remaining 68% stated that the
animal was anaesthetized using pentobarbitone, Hypnorm, Halothane, ether or Ketamine.
The cytochrome P450 enzyme system, which 60% of respondents claimed to study, is
induced by pentobarbitone and may be inactivated by Halothane making these surprising
choices as the method of anesthesia. The buffers used during the perfusion procedure
included Hanks' Balanced Salt Solution, Earl's Balanced Salt Solution and Krebs-Ringer
buffers and 16% claimed not to test the temperature of the solutions regularly during the
perfusion procedure. Three different buffering systems were used, HEPES, HEPES with
sodium bicarbonate and sodium bicarbonate gassed with a mixture of 5% CO₂ and 95%
O₂. 18% did not check the pH of the medium regularly during the procedure. The agent
employed to dissociate the organ into a cell suspension also varied. Although the majority
of respondents employed collagenase, 28% also used an EGTA pre-perfusion and one respondent used EGTA alone. The concentration of the collagenase used varied from 0.03% (w/v) to 0.14% (w/v) and the length of time for rodent liver to be dissociated into single cells varied from 10 to 60 minutes. The entire cell isolation and washing procedure was found to take from 30 to 120 minutes and to yield hepatocytes with a viability of greater than 80% (92% of respondents). Although there were 29 respondents to this survey, they reported 50 different isolation procedures indicating that even within laboratories, there is variation in the methods employed to isolate hepatocytes. In an attempt to answer this variation, ECVAM (European Center for the Validation of Alternative Methods), after a workshop attended by those working with hepatocytes in both academia and industry, published a set of recommendations (Blaauboer et al. 1994) which include the following:

- A two step perfusion method should be used
- A flow rate of 40 ml/min should be used
- The perfusion should be carried out at 37°C
- All solutions should have a pH of 7.4 and an osmolarity of 300 mOsm/litre
- Dispersion of the hepatocytes should be carried out in culture medium containing 5-10% fetal calf serum (FCS) or 1% bovine serum albumin (BSA) and at the temperature the isolated cells will be used

Prior to their use for any purpose, isolated hepatocytes should be extensively characterised. Assays for viability include the study of cellular morphology, dye exclusion assays, determination of cellular ATP content and the extent of release into the surrounding medium of cellular enzymes. Lactate dehydrogenase (LDH) is one of the
most commonly used enzymes as its release occurs quickly after damage to the cell. Cell quantity may be determined by both staining and counting and the assessment of DNA content. Gluconeogenesis and cytochrome P450 function denote the prevalence of liver-specific function. Other parameters include cellular protein and lipid content, membrane phospholipid content and a broad range of intracellular and membrane-bound enzyme activities.

The method for hepatocyte isolation that was determined during the ECVAM workshop described previously has been adopted by the Hepatocyte Users Group (HUG). This chapter describes the characterisation of rat hepatocytes isolated using this method and evaluates their suitability for use as a research tool. Following isolation and characterisation, these hepatocytes will be used in suspension cultures to study the effects of xenobiotics (oxysterols) on the antioxidant defense system and the possible protective effects of naturally occurring phyto- and agrochemicals. Suspension cultures were chosen over a more long-term culture system due to the complexity of maintaining differentiated hepatocytes in such a long-term culture system. Hepatocytes may be successfully maintained in suspension cultures for 4-6 hours and as the cell architecture and intercellular contacts which may be established during long-term culture are not of primary importance in the studies undertaken, a long-term culture system was not deemed necessary.

To assess the suitability of hepatocytes as a model for studying the effects of some food components on the antioxidant defense system, a study with the following objectives was undertaken:

- To prepare rat hepatocytes using the isolation procedure recommended by ECVAM.
• To study freshly isolated cells with respect to morphology, viability, protein, DNA and ATP content

• To determine whether these cells retain liver specific function by measuring the extent of gluconeogenesis, protein synthesis and cholesterol synthesis

• To determine baseline levels of activity of the cellular antioxidant enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) so that the effects of food components on these enzyme levels may be studied

• To determine the extent of lipid peroxidation in control cells, again so that the effects of some food components on peroxidation of liver cell membranes may be studied

2.2 Reagents

Hanks' Balanced Salt Solution, collagenase from *Clostridium histolyticum* type 1A, Ham's F12 nutrient medium, Percoll, trypan blue solution, 7-ketocholesterol, cholestanetrol, hydrogen peroxide, thiobarbituric acid, trichloroacetic acid, NADH, pyruvic acid, BSA solution (fraction V), DNA standard (from calf thymus), bisbenzimidazol, ATP, glucose, glucose-6-phosphate dehydrogenase, hexokinase, glucose kit, imidazole, glucose-6-phosphate, NADPH, 3-hydroxy-3-methylglutaryl-CoA were purchased from Sigma Ltd, London. Ransod and Ransel kits for the determination of SOD and GPx activity were purchased from Randox Laboratories Ltd, Armagh. [3H] mevalonolactone (33 Ci/mmol), 3-hydroxy-3-methyl[3-14C]glutaryl-CoA (45 6 mCi/mmol), L-[4,5-3H]leucine (148 Ci/mmol, distribution of label is as follows 86.7% at the 5-H position and 13.3% at the 4-H position) and NCS II tissue solubiliser were purchased from Amersham, Canada Ltd. Ecolite scintillation fluid was purchased from
ICN Biomedicals, California. All other reagents were purchased from BDH, Poole and Riedel de Haen, Irl.

**Animals**

The animals used were Sprague Dawley rats, fed standard rat chow.

### 2.3 Methods

#### 2.3.1 Hepatocyte Isolation (Blaauboer et al, 1994)

Sprague Dawley rats were sacrificed via Halothane anaesthesia followed by cervical dislocation. The caval and portal veins were then cannulated using 14 gauge cannulas and an *in situ* two-step collagenase perfusion was carried out. This system is shown diagrammatically in Figs 2.3.1a and 2.3.1b. Initially, 400 ml of calcium-free Hanks' Balanced Salt Solution (BSS) containing 0.1 mM EGTA was pumped through the liver over a period of 10 minutes and allowed to run to waste. Following this, 80 ml of calcium and EGTA-free Hanks' BSS was pumped through the liver to remove any remaining EGTA. A recirculating perfusion was then set up using 150 ml of a 0.05% (w/v) collagenase solution in Hanks' BSS containing 1 mM calcium. The perfusion was stopped when the liver had softened to such an extent that the collagenase solution was seen to leak from the liver lobes. This usually took between 7 and 10 minutes. The liver was then excised and incubated for 15 minutes in the collagenase solution. To remove any extracellular material, the liver was passed through a nylon mesh with a Hanks' BSS.
Fig 2.3.1a Diagrammatic representation of liver perfusion

Reproduced from Berry et al (1991)
Liver perfusion apparatus, showing temperature control (water bath maintaining perfusion solutions at 37°C), bubble trap, dissection table, pump, waste container and tubing. Blue tubing is that going to the liver. Red tubing is that going from the liver to waste — in order to flush out blood and finally, purple tubing is that leading from the liver to the perfusion solution container in order to recirculate the collagenase solution.
solution containing 0.5% (w/v) BSA and the cells washed three times in phosphate buffered saline (PBS) Percoll was added to the cell suspension and this mixture was centrifuged for 10 minutes at 800 rpm in a Heraeus centrifuge. The cells at the top of the supernatant (non-viable) were removed and a further two washes were carried out on the remaining cells. Finally, the cells were suspended in 50 ml PBS and were then ready for characterisation and use. A flow diagram depicting this process is given in Fig. 2 3 1c.

2.3.2 Assessment of Yield and Viability using Trypan Blue Exclusion (Blaauboer et al., 1994)

20 µl cell suspension was diluted with 130 µl PBS and added to 50 µl 0.4% (w/v) trypan blue. The cells were then counted in a haemocytometer, a blue colour indicating the non-viable cells. The haemocytometer chamber holds 1 x 10^-4 ml and therefore the cell count obtained was multiplied by the dilution factor (10) and by 10^4 to determine the number of cells/ml.

2.3.3 Assessment of Cell Viability using LDH Leakage (Racher, 1995)

An aliquot of cell suspension containing approximately 10^7 cells/ml was spun down and the supernatant (supernatant) removed and kept for analysis. The cell pellet was then resuspended in 1 ml PBS and lysed via incubation in 0.15% (w/v) Triton X100 for 5 minutes. The extract was then spun down and the resulting supernatant (cellular pellet) removed and kept for analysis. The samples were either examined for LDH activity immediately or kept at -20°C until analysis was carried out.
Procedure for **In Situ** Recirculating Perfusion

**Sprague Dawley rat (250 g)**
sacrifice via halothane anesthesia and cervical dislocation

\[\downarrow\]

**Cannulation**
of portal vein (14g) and inferior vena cava (14g)

\[\downarrow\]

**Two step perfusion**
1. Hanks' BSS containing EGTA
2. Hanks' BSS containing calcium and collagenase

\[\text{pH 7.4, temperature 37°C}\]

\[\downarrow\]

**Removal of Debris**
Filter through nylon mesh with protein-containing Hanks' BSS

\[\downarrow\]

Wash X 3 in PBS

**Percoll treatment**

\[\downarrow\]

Resuspend in 50 ml PBS
0.833 ml of a 0.24 M βNADH solution, prepared in 81.3 mM Tris buffer (pH 7.2), was mixed with 0.07 ml of the supernatant or cellular pellet sample in a cuvette. The background change in absorbance at 340 nm was monitored against air until a stable reading was reached. After addition of 0.167 ml of a 9.76 mM pyruvate solution prepared in buffer the absorbance due to the conversion of βNADH to βNAD in the following reaction

\[
\text{Pyruvate} + \beta\text{NADH} \rightarrow \text{lactate} + \beta\text{NAD}
\]

was monitored for three minutes at 30°C.

LDH activity was calculated using the following equation:

\[
\text{Activity} = \left[ \frac{\Delta A_{rxn}}{\Delta t} - \frac{\Delta A_{bkgd}}{\Delta t} \right] \times \frac{V_t}{6.22 \times 1 \times V_s}
\]

Where \(\Delta A_{rxn}\) = change in absorbance after addition of pyruvate

\(\Delta t\) = time period during which this change in absorbance occurred

\(\Delta A_{bkgd} / \Delta t\) = the same as above but for the background reading

\(V_t\) = the total volume of the assay mixture in mls

\(V_s\) = the volume of the supernatant or cellular sample in mls

\(l\) = the pathlength in cm

6.22 l mmol\(^{-1}\) cm\(^{-1}\) = mmolar extinction coefficient for βNADH

The expression of LDH activity in the cellular pellet as a percentage of the LDH activity in the total sample (both supernatant and cellular pellet) gave an indication of percentage.
cell viability, 1 unit of LDH activity is defined as 1 μmol of NADH consumed per minute at 30°C

2.3.4 Determination of the Optimum Method for Cell Lysis

To determine the optimum procedure for cell lysis without damaging the intracellular contents, cells were exposed to different durations of cell sonication and of differing concentrations of Triton X100. The resulting LDH activity was measured as described in section 2.3.3. 1 ml aliquots of freshly isolated cell suspension in PBS were subjected to either sonication on ice for 0 to 240 seconds or were exposed to 0, 0.01, 0.05, 0.1, and 0.15% Triton X100 for 1, 3, 5, and 10 minutes. The cellular debris was removed via centrifugation and the resulting supernatant assayed for LDH activity.

2.3.5 Lowry Method for Protein Determination, as Modified by Gibson and Skett, (1994).

The cell sample was diluted 1 in 100 with 0.5 M NaOH. 0 to 1 ml aliquots of this 1/100 dilution were further diluted to 1 ml in 0.5 M NaOH. 1 ml standards containing 0 to 100 μg BSA were also prepared in 0.5 M NaOH. 5 ml of a copper reagent composed of a 100:1:1 mix (v:v:v) of 2% (w/v) sodium carbonate in 0.1 M NaOH, 1% (w/v) copper sulphate in water and 2% (w/v) potassium tartrate in water was added to both samples and standards, mixed and incubated at room temperature for 10 minutes. 0.5 ml of 1 M Folin – Ciocalteau reagent was then added and, after mixing, the tubes were incubated for 30 minutes at room temperature. The absorbance at 750 nm was then
measured and the protein concentration of the cell sample determined from a standard curve.

2.3.6 Assessment of DNA Content (Bonis et al, 1991)

An aliquot of cell suspension containing approximately $10^7$ cells/ml was spun down and resuspended in 4 ml 0.07 M phosphate buffer pH 7.4, containing 2 M NaCl and 0.2 M EDTA. The absorbance at 356 nm was measured and the sample diluted so that the absorbance was less than 0.2. This ensured that the fluorescence measured in the assay was not affected by turbidity.

DNA standards, 0 to 16 μg, were prepared in 1 ml 0.07 M phosphate buffer pH 7.4 containing 2 M NaCl. 1 ml 0.02 mg/ml bisbenzimidazol (Hoechst dye no 33258), prepared in the same buffer, was added to the standards and to 1 ml aliquots of the diluted cell sample. These solutions were then incubated in the dark for 1-2 hours with constant mixing. Fluorescence was measured with a Perkin Elmer Luminescence Spectrophotometer L550, with an excitation wavelength of 356 nm and an emission wavelength of 458 nm. Slit widths of 10 nm (excitation) and 7.5 nm (emission) were used. The DNA concentration of the cell sample was determined from a standard curve.

2.3.7 Assessment of ATP Content (Pieczonka and Dehn, 1993)

An aliquot of cell suspension containing approximately $10^7$ cells/ml was lysed by incubating in 0.15 % (w/v) Triton X100 for 5 minutes and spinning down the cellular debris which was discarded. Standards containing 0 to 0.5 mM ATP were prepared. 333 μl sample or standard was mixed with 666 μl working reagent, composed of 50 mM
Trizma buffer pH 7.6, 12.4 mM NADP⁺, 620 mM glucose, 125 mM MgCl₂ and 250 U glucose-6-phosphate dehydrogenase, in a cuvette. The absorbance was measured at 340 nm. 166.5 μl hexokinase (70 U/ml) was then added to each cuvette and mixed for 15 minutes. The absorbance at 340 nm was again measured. The absorbance of the ATP was calculated as follows:

\[
\text{(Sample absorbance}_{15} - \text{sample absorbance}_0) - (\text{blank absorbance}_{15} - \text{blank absorbance}_0).
\]

The ATP concentration of the samples was determined from a standard curve.

2.3.8 Assessment of Glucose Production (Berry et al, 1991)

After isolation, cells from fasted rats were incubated for three hours at 37°C, 5% CO₂ and > 80% relative humidity in 5 ml of a glucose-free medium. The medium was composed of 78.7 mM NaCl, 3.1 mM KCl, 0.8 mM KH₂PO₄, 0.8 mM MgSO₄, 1.7 mM CaCl₂, 16.7 mM NaHCO₃ and 0.5% (w/v) BSA. Each incubation contained 20 mM lactate and 2 mM pyruvate as gluconeogenic precursors. Following incubation cell samples were frozen until the glucose content was assayed using a kit from Sigma. The kit operates on the principle that glucose is converted to 6-phosphogluconate via a coupled enzyme reaction as shown below:

\[
\text{hexokinase} \\
\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} \\
\]

\[
\text{Glucose-6-phosphate-dehydrogenase} \\
\text{Glucose-6-phosphate} + \text{NADP}^+ \rightarrow \text{6-phosphogluconate} + \text{NADPH}
\]
The reduction of NADP to NADPH can be monitored spectrophotometrically at 340 nm. As one mole of NADP is reduced for every mole of glucose converted to 6-phosphogluconate, the change in absorbance can be directly correlated to the glucose concentration using the Beer-Lambert law which states

\[ A = \varepsilon cl \]

Where \( A \) is absorbance, \( \varepsilon \) is molar extinction coefficient, \( c \) is concentration (moles/litre) and \( l \) is the lightpath (cm). As a standard cuvette was used the lightpath was 1 cm. The molar extinction coefficient for NADP\(^+\) is 6220 l mol\(^{-1}\) cm\(^{-1}\).

The glucose reagent, which is composed of 1.5 mM NADP, 1 mM ATP, 1000 units/L hexokinase, 1000 units/L glucose-6-phosphate dehydrogenase, 2 mM magnesium ions and a pH 7.5 buffer, was reconstituted in distilled water. 1.5 ml of the glucose reagent was mixed with 0.01 ml sample or 0.01 ml distilled water (blank). Following a 5 minute incubation at room temperature the absorbance was read at 340 nm using distilled water as reference. The difference in absorbance between the blank and the sample was then used to calculate the glucose concentration with the following equation

\[
\frac{\Delta A \times V_t \times MW}{6.22 \times l \times V_s \times 1000}
\]

where \( \Delta A \) = the difference in absorbance at 340 nm between blank and sample,

\( V_t = \) total volume (ml)

\( MW = \) molecular weight of glucose (180.16)

\( 6.22 = \) mM extinction coefficient of NADP at 340 nm

\( l = \) lightpath (cm)
Vs = sample volume (ml)

1000 = conversion from micrograms to milligrams

To ensure the turbidity of the sample did not affect absorbance readings, 100 μl of the cellular sample was mixed with 1 ml water and the absorbance measured at 340 nm. This reading was then subtracted from the glucose absorbance.

2.3.9 Protein Synthesis (Shultz et al., 1992)

Following isolation, 1 x 10^6 cells were pulsed with L-[4,5-^3H]leucine (5 μCi) for three hours at 37°C and 5% CO₂. Following incubation, cells were washed 3 times with PBS and the supernatant discarded. One ml of 10% (w/v) TCA was added and the samples were kept on ice for 15 minutes. After centrifugation (10 min at 750 g) and removal of the supernatant, the pellet was washed a further 3 times with 10% (w/v) TCA and finally resuspended in 100 μl of NCS II tissue solubiliser. These solutions were digested for 30 minutes at 60°C. The protein extracts were transferred to scintillation vials containing 3 ml methanol and 10 ml Ecolite scintillation fluid. Samples were counted for radioactivity in a Beckman LS6500 scintillation counter.

2.3.10 Determination of Cholesterol Synthesis (Geelen et al., 1991)

Cells (1 ml suspensions) were incubated as before although in this case the medium used was Ham’s F12 nutrient medium. Following incubation, cholesterol synthesis was assessed by measuring the activity of expressed 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase. 100 μl hepatocyte suspension was added to 50 μl assay reagent. The assay reagent comprised 16.7 mM imidazole, 30 mM...
EDTA, 0.3 mM EGTA, 50 mM potassium fluoride, 33.5 mM glucose-6-phosphate, 3.4 mM NADP⁺, 2.2 mM dithiothreitol, 0.33 mM 3-hydroxy-3-methyl[3-¹⁴C]glutaryl-CoA (2 dpm/pmole), 157.5 mU glucose-6-phosphate dehydrogenase and 64 μg digitonin/mg cell protein. This assay mixture was then incubated for 5 minutes at 37°C, 5% CO₂ and >80% relative humidity. Reactions were terminated by the addition of 20 μl concentrated HCl. Blanks were stopped at zero time. The assay mixtures were incubated at 37°C for 30 minutes to lactonise the mevalonate formed during the reaction. [³H] mevalonolactone (2.2 x 10⁴ dpm) was added to all samples as an internal standard. Thin layer chromatography was carried out on all samples to separate mevalonolactone and cholesterol using a 1:1 acetone/benzene solvent mix. The plates were then cut into small pieces and placed in scintillation vials before being counted for radioactivity.

2.3.11 Determination of the Specific Activity of the Antioxidant Enzymes catalase, superoxide dismutase and glutathione peroxidase

*Catalase (Aebi, 1984)*

1 ml cell sample was incubated with 0.15% (w/v) Triton X100 for 5 minutes to lyse the cells. The sample was then spun down to remove any cellular debris. 2.9 ml 50 mM potassium phosphate buffer, pH 7 and 100 μl 0.3 M H₂O₂ were mixed in a quartz cuvette and background absorbance at 240 nm was determined over 30 seconds. 10 μl of the sample was then added and the absorbance read for another 30 seconds. The amount of H₂O₂ reduced, corresponding to the amount of catalase present, was determined using the Beer-Lambert law, A = εcl, where A = absorbance, ε = molar extinction coefficient = 40 l mol⁻¹ cm⁻¹, c = concentration and l = pathlength.
Superoxide dismutase

Cells were lysed as described above. SOD activity was determined using a kit supplied by Randox Laboratories Ltd. The kit utilises xanthine and xanthine oxidase to produce superoxide radicals. These radicals then react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye which absorbs at 505 nm.

Xanthine oxidase

\[
\text{Xanthine} \rightarrow \text{Uric acid} + O_2^- \\
\text{INT} \rightarrow \text{Formazan dye}
\]

The concentration of superoxide dismutase is calculated from the rate of inhibition of this reaction.

1.7 ml of the substrate (containing 0.05 M xanthine and 0.025 M INT) was added to 0.05 ml sample, standard or 0.01 M phosphate buffer, pH 7 (blank). These were mixed and brought to 37°C. 0.25 ml 80 units/L xanthine oxidase was added and 30 s after mixing the initial absorbance A1 was read. The final absorbance A2 was read after a further 3 mins. The change in absorbance per minute was calculated i.e. (A1-A2)/3. The rate obtained using the blank is the rate of the uninhibited reaction and all standard and sample rates were converted to a percentage of this rate and subtracted from 100% to give a percentage inhibition. The equation for this calculation is shown below:

\[
100 - \frac{(\Delta A_{\text{std/min}} \times 100)}{(\Delta A_{\text{blank}})} = \% \text{ inhibition}
\]

\[
100 - \frac{(\Delta A_{\text{sample/min}} \times 100)}{(\Delta A_{\text{blank}})} = \% \text{ inhibition}
\]
The percentage inhibition for each standard was plotted against the Log_{10} of the standard concentration (units/ml). The SOD activity of the samples was determined from this standard curve.

**Glutathione peroxidase**

GPx activity was determined using a kit supplied by Randox Laboratories Ltd. The oxidation of glutathione by organic hydroperoxides is catalysed by glutathione peroxidase. The oxidised glutathione is immediately recycled to the reduced form by glutathione reductase with a concomitant oxidation of NADPH to NADP⁺. This oxidation can be followed spectrophotometrically at 340 nm. The organic hydroperoxide used in this kit was cumene hydroperoxide.

1 ml of the reagent (containing 4 mM glutathione and ≥ 0.5 U/L glutathione reductase) was added to 0.04 ml 4.3 μM cumene hydroperoxide and the mixture was brought to 37°C. 0.02 ml sample or distilled water (blank) was added and, after one minute, the absorbance was measured at 340 nm against air. The absorbance was again measured after 2 and 3 minutes. The GPx concentration was calculated using the equation:

\[ U/L = 8412 \times \Delta A_{340nm} / \text{minute} \]

Where 8412 is calculated using the Beer Lambert law taking into account dilutions, sample and total volumes and light path length.

**2.3.12 Determination of Lipid Peroxidation (Esterbauer and Cheeseman, 1990)**

Lipid peroxidation was assessed by quantifying the level of thiobarbituric acid reactive substances (TBARS) present in the cell samples. Cell samples were lysed as described before. 1 ml of cell lysate was mixed with 2 ml cold 10% (w/v) trichloroacetic acid to precipitate protein. After centrifugation, the resulting supernatant was mixed with
an equal volume of 0.67% (w/v) thiobarbituric acid. This mixture was boiled for 10 minutes. After the mixture was cooled, the absorbance was read at 532 nm. The concentration of TBARS was calculated using the Beer-Lambert law.

2.3.13 Statistics

All results were compared using the Student t Test. n=3 denotes data obtained from three different animals at three different times each of which were carried out in triplicate.

2.4 Results:

2.4.1 Yield, Viability and Morphology

The average yield of liver parenchymal cells was found to be $3.2 \pm 0.5 \times 10^7$ cells/g wet weight. Using trypan blue 0.4% (w/v) exclusion, the average viability was found to be $80.6 \pm 2.7\%$. Using LDH leakage, the average viability was found to be $66.1 \pm 1.5\%$

These two methods of assessing viability were compared and the resulting graph is shown in Fig. 2.4.1a. A linear correlation was found between the two methods with an $r^2$ value of 0.96, (p<0.05). Cells, as shown in Fig. 2.4.1b, were found to be a round, regular shape and to be seen as orange/yellow in colour. Non-viable cells, which had taken up the dye were seen to be blue in colour.

2.4.2 Determination of Optimum Method for Cell Lysis

When sonication was used to lyse the cells, LDH activity, expressed as a percentage of maximum activity increased as sonication time increased. Sonication for
Fig 2.4.1a Correlation Between Trypan Blue Exclusion and LDH Leakage as Methods of Determining Viability

Fig 2.4.1a showing a linear correlation between LDH leakage and trypan blue exclusion as methods of determining viability. The $r^2$ value was found to be 0.96 with 95% confidence.
Viable cells are seen to be orange/yellow in colour and non-viable cells are seen to be blue.
30s released 59.3% of the maximum LDH activity from cells. Increasing sonication time to 60 and 90 s resulted in approximately 10 and 20% more activity respectively being measured. Activity after 120 s (60.4%), although lower than that observed after 90 s, was not significantly different to that observed at shorter sonication times. Maximum LDH activity was observed after 180 s. Activity decreased to 25.7% after sonication for 240 s, indicating a possible damaging effect of prolonged sonication. The LDH activity measured following sonication is shown in Table 2.4.2a.

<table>
<thead>
<tr>
<th>Sonication Duration, seconds</th>
<th>LDH activity as a percentage of maximum activity observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>59.3 ± 3.4</td>
</tr>
<tr>
<td>60</td>
<td>67.7 ± 2.7</td>
</tr>
<tr>
<td>90</td>
<td>72.7 ± 3.3</td>
</tr>
<tr>
<td>120</td>
<td>60.4 ± 5.2</td>
</tr>
<tr>
<td>180</td>
<td>100 ± 2.1 (p&lt;0.05)</td>
</tr>
<tr>
<td>240</td>
<td>25.7 ± 4.6</td>
</tr>
</tbody>
</table>

For all values n=3, errors shown are SEM.

The LDH activity measured following incubation with Triton X100 is shown in Table 2.4.2b. It is apparent that as Triton X100 concentration increased from 0.01% to 0.1%, shorter incubation times were required to achieve maximum LDH release. In the presence...
of 0.01% Triton X100, maximum activity was released after 10 min incubation. In the presence of 0.05% Triton X100, maximum activity was released after 5 min, while incubation for 1 min in 0.1% Triton X100 resulted in maximum activity. Extending the incubation time with Triton X100 (0.05-0.1%) resulted in a marked decrease in enzyme activity, indicating possible lability of the enzyme when exposed to Triton X100 (0.05-0.1%). At higher concentrations (0.15%) of Triton X100, lability was not apparent, maximum activity being observed after 5 and 10 minutes.

Table 2.4.2b LDH Activity in 1 ml Hepatocyte Suspensions as a Percentage of Maximum Activity Observed Following Incubation with Triton X100

<table>
<thead>
<tr>
<th>Incubation Duration, min</th>
<th>[Triton X100] 0.01%</th>
<th>0.05%</th>
<th>0.1%</th>
<th>0.15%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>81.4 ± 3.5</td>
<td>69.5 ± 3.6</td>
<td>100 ± 5.1</td>
<td>65.8 ± 5.8</td>
</tr>
<tr>
<td>3</td>
<td>86.1 ± 5.3</td>
<td>68.3 ± 4.1</td>
<td>73.7 ± 1.1</td>
<td>77.7 ± 3.2</td>
</tr>
<tr>
<td>5</td>
<td>79.6 ± 2.6</td>
<td>100 ± 4.1</td>
<td>67.7 ± 4.3</td>
<td>100 ± 4.5</td>
</tr>
<tr>
<td>10</td>
<td>100 ± 2.3</td>
<td>54.9 ± 6.1</td>
<td>66.5 ± 3.9</td>
<td>98.4 ± 2.4</td>
</tr>
</tbody>
</table>

*For all values n=3, errors shown are SEM*

To determine the overall most efficient method for cell lysis, the level of LDH activity (U/10^5 cells) was compared under the optimum sonication conditions (i.e., three minutes) and under optimum conditions for Triton X100 induced lysis (i.e., 0.01% (w/v) Triton X100 for 10 minutes, 0.05% (w/v) Triton X100 for 5 minutes, 0.1% (w/v) Triton X100 for 1 minute and 0.15% (w/v) Triton X100 for 5 minutes). The results are shown in Fig.
242a LDH activity increased in a step-wise fashion as concentration of Triton X100 increased from 0.01% (0.045 U/10^5 cells), to 0.15% (0.07 U/10^5 cells). Activity was approximately 10 fold higher when cells were lysed in 0.15% (w/v) Triton X100 compared with sonication. Incubation with Triton X100 was found in all cases to be a more effective method of cell lysis than sonication and the optimum conditions were found to be 0.15% (w/v) Triton X100 for 5 minutes.
Fig. 2.4.2a Determination of Optimum Cell Lysis Conditions

Fig. 2.4.2a showing the activity of LDH measured when 1 ml hepatocyte suspensions were lysed using different methods. For all values errors shown are SEM and n=3.
243 Protein, DNA and ATP Content

The average protein, DNA and ATP contents of freshly isolated liver cells are shown in Table 243a.

Table 243a Protein, DNA and ATP contents of freshly isolated rat hepatocytes

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg/10^5 cells</td>
<td>0.19 ± 0.015, n = 10</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>µg/10^5 cells</td>
<td>3.47 ± 0.37, n = 10</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mM</td>
<td>2.02 ± 0.43, n = 8</td>
<td></td>
</tr>
</tbody>
</table>

Errors shown are SEM

The % viability was plotted against protein content, DNA content and ATP content and the results are shown in Fig 243a, 243b and 243c respectively. A linear correlation was found between cell viability and protein content, r^2 = 0.82, p<0.05, and between cell viability and DNA content, r^2 = 0.85, p<0.05. No correlation was found between ATP and % viability. A linear relationship was found between protein and DNA content, r^2 = 0.8, p<0.05 (Fig 243d).
Fig 2 4 3a showing a linear correlation between protein content and % viability as measured by trypan blue exclusion. The $r^2$ value was found to be 0.82 with 95% confidence.
Fig 2.4.3b DNA Content of Cells as a Function of Viability

![Graph showing a linear correlation between DNA content and % viability as measured by trypan blue exclusion. The $r^2$ value was found to be 0.847 with 95% confidence.]

$\text{DNA Content, } \mu\text{g} / 10^5 \text{ cells}$

$\% \text{ Viability as measured by Trypan Blue Exclusion}$

$r^2 = 0.847$

Fig 2.4.3b showing a linear correlation between DNA content and % viability as measured by trypan blue exclusion. The $r^2$ value was found to be 0.847 with 95% confidence.
Fig 2 4 3c ATP Content of Cells as a Function of Viability

Fig 2 4 3c showing the relationship between ATP levels and % Viability. No correlation is seen between the two, the $r^2$ value being 0.276 with 95% confidence.
Fig 2.4.3d DNA Content as a Function of Protein Content

![Graph showing the linear correlation between protein and DNA content of hepatocytes. The $r^2$ value was found to be 0.81 with 95% confidence.](image)

$\text{DNA content, ng} / \text{10}^5 \text{ cells}$

$\text{Protein content, mg} / \text{10}^5 \text{ cells}$

$r^2 = 0.81$

Fig 2.4.3d showing a linear correlation between protein and DNA content of hepatocytes. The $r^2$ value was found to be 0.81 with 95% confidence.
Following incubation at 37° C and 5% CO₂ for 3 hours, hepatocytes in suspension in a glucose-free medium were found to produce 0.04 ± 0.006 mg glucose/mg protein. Under similar conditions, except that glucose was supplied, hepatocytes were found to incorporate 180 ± 11 7 dpm L-[4,5-3H]leucine hour⁻¹ 10⁵ cells⁻¹. The specific activity of HMG CoA reductase in suspension cultures of isolated hepatocytes was observed to be 52.2 ± 9.5 pmoles [¹⁴C] mevalonate min⁻¹ mg⁻¹ protein.

2.4.5 Antioxidant Enzyme Levels and Lipid Peroxidation

Antioxidant Enzyme Levels

The levels of antioxidant enzymes found in hepatocytes after three hours incubation are shown in Table 2.4.5.a.

Table 2.4.5.a: Antioxidant enzyme activities in rat hepatocytes after 3 hour incubation

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity, U/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>64.9 ± 7.3, n=4</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>1.7 ± 0.2, n=4</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>0.4 ± 0.02, n=5</td>
</tr>
</tbody>
</table>
Catalase activity was measured in Bergmeyer units which are defined as µmol [H₂O₂] decomposed min⁻¹ ml⁻¹ at pH 7 and 25°C. The units of GPx activity are defined as µmol NADPH oxidised min⁻¹ at pH 7.4 and 30°C and 1 unit of SOD activity is that quantity of enzyme that inhibits by 50% the conversion of xanthine to a formazan dye by xanthine oxidase.

**Lipid Peroxidation**

After incubation for 3h the extent of lipid peroxidation in the rat hepatocytes as measured by the TBARS method was found to be 1.36 ± 0.09 nmol MDA/mg protein, n=8.

### 2.5 Discussion:

The method of hepatocyte isolation described in this chapter is one that has been recommended by both ECVAM and by HUG, agreed on by experts in the field of hepatocyte research but not extensively studied in practice. An international inter-laboratory study to validate this method has been delayed and therefore the aim of this project was to isolate rat hepatocytes using this method, subject them to a battery of tests with which to assess their structural and biochemical integrity before using them as a research tool to study the hepatic effects of some food components. The contents of this chapter encompass the study of the isolated hepatocytes and the assessment of their validity for use. A standard set of characterisation assays has not yet been recommended but should include a study of morphology, viability and biochemical function, including specific liver function.

Prior to carrying out many of the characterisation assays it was necessary to lyse cells and release the intracellular contents. It was necessary to ensure that this was carried
out in such a way as to ensure complete lysis but to avoid damage to the released contents. Two methods of cell lysis were compared: sonication using a B Braun Labsonic sonicator and exposure to the detergent Triton X100. Examination of Table 2.4.2a shows an increase in LDH activity up to 180 seconds of sonication but then a large decrease to 24.8% of this maximum activity when cells were exposed to a further 60 seconds of sonication. Exposure to varying concentrations of Triton X100 yielded a similar pattern, of an increase in activity to a maximum followed by a decrease in activity when cells were exposed to Triton X100 for a longer period of time (Table 2.4.2b). A comparison of sonication and Triton X100 as methods of cell lysis is shown in Fig. 2.4.2a. Exposure to Triton X100 is seen to be more effective in all cases than sonication and the optimum conditions for cell lysis were found to be exposure of cells to 0.15% (w/v) Triton X100 for 5 minutes. This method was used to lyse hepatocytes in all subsequent experiments.

LDH activity was used to measure the effectiveness of the lysis procedure as it is a cytosolic enzyme and therefore quickly released. A decrease in the activity of this enzyme would indicate that, although the cellular contents of the cell have been released, the lysis procedure was damaging the released cellular components and should therefore be curtailed. The optimum lysis conditions would be those that yielded the highest LDH activity as that would indicate sufficient cell lysis but minimum damage to the cellular contents through continued exposure to the lysis procedure.

Morphology was studied using standard light microscopy with the aid of trypan blue staining to distinguish between viable and non-viable cells. The isolated cells, as seen in Fig. 2.4.1b, were a pale orange in colour and had a smooth round shape (viable cells). Non-viable cells become blue in colour, following uptake of the trypan blue dye.
have a granular cytoplasm and can show signs of blebbing or swelling. It is not necessary to stain with trypan blue to distinguish cells showing these signs of damage. Severely damaged cells were removed during the isolation procedure through centrifugation with Percoll. Percoll is a colloidal suspension of silica particles coated with polyvinylpyrrolidone and is unable to enter either intact or damaged cells. Damaged cells, which have taken up water, are less dense than intact cells and will float on top of the Percoll suspension following centrifugation. Intact cells will pellet making them easy to isolate (Berry et al., 1991). One recommendation of the ECVAM report with respect to characterisation of the resulting hepatocytes is that the viability following isolation should be at least 80% using trypan blue exclusion in a protein-free medium (Blaauboer et al., 1994). The average viability obtained using this method of isolation was 80.6 ± 2.7% therefore meeting this requirement. Much has been published concerning the best method for determining viability. Trypan blue exclusion, although the most widely used method, has many drawbacks. The cell count must be completed between three and six minutes of addition of the dye and it is essential that no protein be present in the medium as trypan blue-protein complexes form and these complexes are too large to enter the cell (Black and Berenbaum, 1964). Baur et al. (1975) suggest that trypan blue exclusion indicates only severe irreversible damage of cells. Cook and Mitchell (1989) state that the initial sites of many cytotoxic agents are intracellular and may require time for expression, therefore trypan blue exclusion would underestimate the extent of the damage. Rather than viability, Seglen (1976) states that trypan blue exclusion measures gross structural integrity at the time of testing.
A second method of determining viability is the leakage of enzymes that are situated in the cytosol. The leakage of lactate dehydrogenase, LDH, was studied. The average viability of the isolated hepatocytes using this method was 66.1 ± 15%. Although considerably lower than the corresponding value obtained using trypan blue exclusion, when the two methods of determining viability were plotted (Fig 2.4 1a) a correlation between the two was found (p<0.05). A choice between the two methods cannot be made as the arguments against trypan blue exclusion, as mentioned earlier, apply equally to LDH release as both rely on membrane integrity. Berry et al. (1991) recommends the use of trypan blue exclusion to determine the initial viability of isolated cells and the use of LDH release when taking viability data from cells in culture over a period of time. Bridges (1981) suggests that there is no ideal method for determining viability and the particular viability assay should be chosen to suit the system being studied.

A protein content of 0.19 ± 0.015 mg/10^5 cells was obtained which agrees with that found by Berry et al. (1991) who reported a level of 0.17 mg/10^5 cells for fed rats. Berry et al. (1991) obtained a value of 1.7 μg DNA per 10^5 cells which is 50% of the value obtained in the present study of 3.47 ± 0.37 μg DNA per 10^5 cells. This difference could be explained by the fact that although the method used to determine protein content used in this study and that used by Berry et al. (1991) were the same (a version of the Lowry technique), the methods used to determine DNA content differed. Whereas a fluorescent technique, specific for double stranded DNA, developed by Boms et al. (1991) was used in this study, Berry et al. (1991) employed the Burton method (Burton, 1956) which involves spectrophotometric determination of the coloured product formed through
reaction of the isolated DNA and diphenylamine Bomis et al (1991) reported higher levels of DNA measured using this method than using other methods, possibly due to DNA loss during the extraction necessary to assess DNA content using other techniques. A second difference involves the animals used. Berry's data was obtained from Hooded Wistar rats where as Sprague Dawley rats were used in this study. Nutritional status of the donor animal has not been found to affect DNA content although protein content can decrease by 3-10% during fasting (Berry et al, 1991).

A linear correlation was found between the protein and DNA contents of the hepatocytes (p<0.05) (Fig 2.4.3d) and also between protein content and viability (p<0.05) (Fig 2.4.3a) and DNA content and viability (p<0.05) (Fig 2.4.3b) This indicates the loss of protein and genetic material from damaged cells. No correlation was seen between ATP content and % viability (Fig 2.4.3c) although the average ATP concentration of 2.02 mM agreed with that found by Berry et al (1991) of 2.42 mM for fed rats. ATP concentration has been used as an indicator of cellular integrity as the ATP content of cells tends to drop rapidly when they are damaged. However, it has been found that damaged cells may maintain a normal ATP/ADP ratio (Berry et al, 1991). A study of the cells removed by Percoll treatment revealed that these cells are devoid of ATP and Berry postulates therefore that an inverse relationship may exist between the percentage of damaged cells and ATP concentration in the hepatocyte suspension. However, if severely damaged cells have been removed by Percoll treatment and the remaining damaged cells still retain a normal ATP/ADP ratio, then it is likely that a correlation between % viability and ATP content will not exist. The data obtained in this study supports this theory.
Both biochemical and liver specific function were assessed using protein synthesis, HMG CoA reductase activity, as the rate limiting enzyme marker of cholesterol synthesis and glucose production. The isolated hepatocytes were found to be biochemically functional and to retain liver specific function. Protein synthesis was found to occur ($180.06 \pm 11.65$ dpm L-[$4,5-^{3}\text{H}$] leucine were incorporated hour$^{-1}$ per $10^5$ cells). Leucine was used as the precursor for protein synthesis as it is not synthesized by hepatocytes and therefore the radio-labeled leucine should not be in competition with endogenous leucine. The rate of catabolism of leucine is not significant and at concentrations above 5 mM, it is possible to equate the specific activity of the radio-labeled leucine with that of the aminoacyl-t-RNA pool (the intracellular precursor pool for protein synthesis) (Berry et al., 1991). Protein synthesis is pH dependent. A decrease in the rate of protein synthesis may be viewed as an indicator of cell injury, possibly more sensitive than LDH release. Goethals et al. (1984) found that paracetamol inhibited the rate of protein synthesis at concentrations that had no effect on LDH release. Protein synthesis can therefore be used to determine harmful effects of xenobiotics. Cholesterol synthesis was found to occur ($52.22 \pm 9.48$ pmoles mevalonate was produced minute$^{-1}$ mg protein$^{-1}$). Cholesterol synthesis was determined by measuring the activity of HMG CoA reductase, the rate-determining enzyme in cholesterol synthesis. The usual method for studies on this enzyme involves the preparation of microsomes, a time-consuming process. In this study, a procedure developed by Geelen et al. (1991) was used. Digitonin was used to permeabilize the cells and expose this endoplasmic reticular enzyme to the assay components. Thus the enzyme was assayed more or less in its natural environment using an assay that was very rapid, so reducing the risk of loss of enzyme function.
Digitonin functions by binding specifically to cholesterol in the cell membrane and forms an insoluble complex which leads to membrane defects (Berry et al, 1991). To determine the optimal concentration of digitonin to use, Geelen et al (1991) exposed hepatocyte cultures to increasing concentrations of digitonin and found that 64 μg digitonin/mg cellular protein yielded optimum results. At this concentration arylesterase, a marker enzyme for the endoplasmic reticulum was only slightly released from the cells, indicating that endoplasmic reticular enzymes would be assayed in situ (Geelen et al, 1991). Doubling the concentration of digitonin was found to have no effect on solubilized or microsomal HMG CoA reductase (Eilenberg et al, 1991). HMG CoA reductase activity in control hepatocytes from Wistar rats after 20 mm incubation was found to be 466 ± 322 pmol min⁻¹ mg⁻¹ protein (Geelen et al, 1991). The activity of HMG CoA reductase in control hepatocytes from Sprague Dawley rats after a three hour incubation in this study was found to be 52.2 ± 9.5 pmol min⁻¹ mg⁻¹ protein. The 10-fold difference in activity could be due to the diurnal variation in HMG CoA reductase activity. The animals used in this study were kept under normal lighting conditions. Geelen et al (1991) do not specify the light conditions used although animals used by Geelen in previous studies on HMG CoA reductase were kept under reverse lighting conditions and sacrificed during the dark cycle (Ingebritson et al, 1979). Activity of this enzyme peaks during the dark cycle (White et al, 1984) due to a peak in the level of circulating insulin which also has a diurnal rhythm and which peaks approximately four hours into the dark cycle corresponding with the highest intake of food in rodents (Easom and Zammit, 1985). The rats used in this study were sacrificed in the morning, during the light cycle. It is to be expected, therefore, that activity measured would be considerably less than that.
from hepatocytes harvested during the dark cycle. Gluconeogenesis was found to occur at a rate of $0.2 \pm 0.02 \mu g$ glucose produced min$^{-1}$ mg$^{-1}$ protein. Glucose production has been used as a means to study metabolic regulation as well as an indicator of cellular integrity. Lactate was used to stimulate gluconeogenesis. Optimum glucose production has been found to occur when lactate/pyruvate levels are at 10:1 (Berry et al., 1991). Hepatocyte suspensions in this study were exposed to such concentrations of these precursors in order to ensure maximum glucose production. Berry et al. (1991) suggests that hepatocytes from fasted rats should be able to produce $0.45 \mu g$ mg$^{-1}$ min$^{-1}$ glucose from lactate. This is approximately double the quantity of glucose detected in this study. The differences seen could again be due to differences in animal strain.

Since suspension cultures of rat hepatocytes were to be used as a tool for studying the effects of micro-food components on the antioxidant defense system of liver cells, it was necessary to determine the baseline activity levels of the antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase and the extent of lipid peroxidation in control hepatocytes. Catalase activity was found to be $64.9 \pm 7.3$ Bergmeyer units/mg protein, a value which correlates with that published by Vasavi et al. (1994) of $57.05 \pm 10.84$ Bergmeyer units/mg protein. A higher concentration of glutathione peroxidase activity was found in this study ($0.41 \pm 0.02$ U/mg protein) compared to $0.095 \pm 0.00612$ U/mg protein (Vasavi et al., 1994). The extent of lipid peroxidation in rat hepatocytes was similar to that of rat liver $1.36 \pm 0.09$ nmol MDA/mg protein was observed in the hepatocytes isolated in this study whereas in the study carried out by Vasavi et al. (1994), $1.1 \pm 0.18$ nmol MDA/mg protein was found. The SOD activities cannot be compared as Vasavi et al. (1994) used the inhibition of epinephrine.
autoxidation to determine SOD activity. The differences in GPx activity seen could be due to the difference in the strain of rats used. Vasavi et al. used Wistar rats whereas the rats used in this study were Sprague Dawley.

2.6 Summary:

Rat hepatocytes isolated using the method recommended by ECVAM were characterised as to structural integrity, using trypan blue dye exclusion and LDH release, physical parameters (DNA and protein content), biochemical integrity (ATP content and protein synthesis) and the retention of liver specific function (gluconeogenesis and cholesterol synthesis). The hepatocytes were found to be sufficiently viable (>80%) and to retain biochemical function (hepatocytes were 2.02 ± 0.46 mM ATP and were found to incorporate 180.06 ± 11.65 dpm L-[4,5-3H] leucine into protein hour⁻¹ per 10⁵ cells). A gluconeogenic rate of 0.04 ± 0.006 mg glucose production/mg protein and HMG CoA reductase activity of 52.2 ± 9.5 pmol [14C] mevalonate min⁻¹ mg⁻¹ protein indicated the retention of liver specific function. The levels of antioxidant enzymes and of lipid peroxidation were found to be quantifiable. Therefore, rat hepatocytes isolated using this method would seem to be suitable for use as a research tool in the study of the effects of micro-food components such as 7-ketocholesterol, cholestanetriol, Cov-ox and conjugated linoleic acid (CLA) on the oxidative status of liver.
CHAPTER 3

The Effect of Cov1-ox on the Antioxidant Defense System in Rat Hepatocytes Challenged with 7-Ketocholesterol and Cholestanetriol
3.1 Introduction

Oxidized derivatives of cholesterol, known as oxysterols, are present in the diet as contaminants of cholesterol-containing foods (Pamangvait et al., 1995). 7-Ketocholesterol and cholestanetanol are two commonly occurring oxysterols formed as a result of B-ring oxidation of cholesterol during processing, preparation and storage of a range of animal food products (Huber et al., 1995, Rose-Salln et al., 1995, Kowale et al., 1996, Nielsen et al., 1996, Zunm et al., 1996, McCluskey et al., 1997, Shozen et al., 1997).

Numerous cellular studies have implicated cholestanetanol and 7-ketocholesterol in membrane and enzyme alterations that are interrelated with the atherogenic effects of oxysterols (Mahfouz et al., 1995, 1996, Verhagen et al., 1996, Gelissen et al., 1996). Feeding a 0.2% mixture of COPs to rats has been reported to modulate age-related changes in hepatic lipid metabolism via changes in HMG CoA reductase, cholesterol 7α-hydroxylase, delta 6 desaturase and lipid peroxidation (Osada et al., 1995). Injection of COPs (5 mg/kg), containing 7-ketocholesterol and cholestanetanol into rats and rabbits has been found to lead to accelerated atherosclerosis much more effectively than with cholesterol itself (Imai et al., 1976, Taylor et al., 1979, Peng et al., 1982, 1985). Jacobsen (1987) reported a possible link between dietary COPs intake and the unusually high incidence of myocardial infarction in the London and West Indian Asian populations that consume ghee, a cooking oil produced from butter under optimum oxidising conditions. The structures of these two COPs are shown in Fig. 3.1.1.
The observation that humans efficiently absorb COPs across the intestine has led to concern about the levels of COPs found in foodstuffs (Emanuel et al, 1991) Strategies to diminish oxysterol levels or their pathological effects may prove useful in enhancing the safety of the food supply. Antioxidant inhibition of cholesterol oxidation in processed foods has been reported using a tocopherol blend (Huber et al, 1995, Li et al, 1996,
Shozen *et al*, 1997) By analogy with food, antioxidants including selenium and α-tocopherol are being investigated as necessary supplements in tissue culture media to protect viability of cells in general and in particular cells that are oxidatively challenged (Leist *et al*, 1996, Ohtani *et al*, 1996) Tocopherol has been found effective in protecting against oxidative stress *in vitro* and *in vivo* and no adverse effects were found when it was fed to rats (400 mg/kg, Vasavi *et al*, 1994)

Exposure of humans to COPs is most commonly through foods and the oxysterols absorbed in the stomach and intestine may be transported to the liver in significant concentrations where they may exert adverse oxidative effects on receptors, proteins or key enzymes regulating metabolism or cellular defense. *In vivo* origins of oxysterols also exist. Cholesterol itself is oxidised via a range of tissue hydroxylases the products of which regulate the expression of the cytochrome P450 gene superfamily involved in sterol metabolism (Stromstedt *et al*, 1996). Formation of 7-ketocholesterol in liver has been reported as a result of liver microsomal NADPH-dependent biotransformation of toxic alkanes (Fanelli *et al*, 1995). Thus, oxysterols of either dietary or biotransformation origin as a result of xenobiotic metabolism might be expected to induce significant biological responses via their direct or free-radical mediated effects on cytochrome P450 sterol regulatory enzymes.

Primary cultures of rat hepatocytes are increasingly being used for investigating the direct effects of phytochemicals on liver cell metabolism and viability. Many studies have demonstrated that chemoprotective and antiatherosclerotic effects of phytochemicals are mediated by specific effects on liver cell metabolism (Khan *et al*, 1992, Gebhardt and Beck, 1996). In particular, antioxidants such as polyphenols,
flavonoids and Vit E in plant foods have been shown to exert hepatoprotective effects against oxidative stress induced by lipid hydroperoxides (Gebhardt, 1993, 1995). Important reasons for the popularity of this *in vitro* system are the increase in our knowledge of technical aspects of cell isolation and culture and the relative ease of obtaining a homogeneous preparation consisting of a single cell type (Blaauboer *et al.*, 1994). The presence of high activity biotransformation enzymes makes this cell type a potential target for reactive intermediates which may mediate hepatotoxicity of steroids in food. The hepatocyte, therefore, may serve as a suitable model with which to investigate the oxidative potential of oxysterols in liver and the protective efficacy of tocopherol.

The enzymatic system regulating oxidative defense in cells and tissues consists of superoxide dismutases (SOD), catalase and glutathione peroxidase (GPx). SOD is thought to be the first line of defense, dismuting the superoxide radical to hydrogen peroxide and water. The end product of SOD action, hydrogen peroxide, is itself very reactive and so either catalase or GPx work with SOD to remove hydrogen peroxide.

The aims of this study were to investigate whether the cholesterol oxidation products, 7-ketocholesterol and cholestanetriol are themselves causative of oxidative stress such as to induce lipid peroxidation of hepatic cellular membranes. The hypothesis that Covi-ox, a blend of mixed tocopherols, accepted worldwide as a safe and effective natural food antioxidant, may also protect cells and tissues from the damaging effects of cholesterol oxides was also tested. The specific objectives were as follows.
• To assess the effect of 7-ketocholesterol, cholestanetriol and Covi-ox on cellular integrity using the parameters discussed in chapter 2, i.e. trypan blue exclusion, LDH release and ATP content

• To assess the effect of 7-ketocholesterol, cholestanetriol and Covi-ox on liver specific function as denoted by protein synthesis and gluconeogenesis

• To determine the levels of the antioxidant enzymes SOD, catalase and GPx following incubation of hepatocytes with 7-ketocholesterol and cholestanetriol in the presence and absence of Covi-ox

• To determine the extent of lipid peroxidation in hepatocytes, as measured by TBARS, following incubation of hepatocytes with 7-ketocholesterol and cholestanetriol in the presence and absence of Covi-ox

3.2 Reagents

Collagenase from Clostridium histolyticum type 1A, Hanks BSS, Hams F12, Percoll, trypan blue solution, 7-ketocholesterol, cholestane 3β, 5α, 6β triol, hydrogen peroxide, thiobarbituric acid and trichloroacetic acid were purchased from Sigma Ltd, London. Ransod and Ransel kits for the determination of SOD and GPx activity were purchased from Randox Laboratories Ltd, Armagh. L-[4,5-3H]leucine (148 Ci/mmol, distribution of label is as follows 86 7% at the 5-H position and 13 3% at the 4-H position) and NCS II tissue solubilisor were purchased from Amersham, Canada Ltd. Ecolite scintillation fluid was purchased from ICN Biomedicals, California. All other reagents were purchased from BDH, Poole and Riedel de Haen, Irl. Covi-ox was kindly donated by Henkel Irl.
Ltd and is composed of approximately 30% tocopherols in powdered form. There is a high proportion of \( \beta, \gamma \) and \( \delta \) tocopherol and a low proportion of \( \alpha \) tocopherol.

### 3.3 Methods

#### 3.3.1 Incubations

20000 ppm solutions of 7-ketocholesterol and cholestanetriol were prepared in ethanol. A 20000 ppm suspension of Covix was prepared in ethanol. Aliquots of these solutions which would give 0 to 2000 ppm of the oxysterol or antioxidant in 5 ml of solution were transferred to 6 well plates where the ethanol was allowed to evaporate. This resulted in 6 well plates with a coating of oxysterol ± Covix and no organic solvents. For control experiments, ethanol alone was added to the wells and allowed to evaporate. Hepatocytes (approximately \( 10^7 \)) were incubated, in these plates, in 5 ml Ham's F12 nutrient medium for 3 hours at 37° C, 5% CO\(_2\) and > 80% relative humidity.

#### 3.3.2 Viability

Viability of the hepatocytes following incubation with Covix, 7-ketocholesterol, cholestanetriol and the oxysterols coincubated with Covix was determined using both trypan blue exclusion and LDH leakage as described in chapter 2.

#### 3.3.3 ATP, Gluconeogenesis and Protein Synthesis

These parameters were determined as set out in chapter 2.
3.3.4 Antioxidant Enzyme Levels and Lipid Peroxidation

The specific activities of catalase, SOD and GPx were determined using the procedures set out in chapter 2. Lipid peroxidation was determined using the TBARS method as described in chapter 2.

3.4 Results

3.4.1 Viability

*Covi-ox*

The Effect of *Covi-ox* on cell viability is shown in Fig 3.4.1a. A difference is seen in the viability as measured by both LDH and trypan blue exclusion although the trends are the same. *Covi-ox* was seen to have no significant effect on hepatocyte viability after 3 hours incubation although the viability was measured as 75.5 ± 4.5% using trypan blue exclusion and 50.6 ± 4.2% using LDH leakage in control cells. Although no significant difference in viability was found after a 3 hour incubation with any of the concentrations of *Covi-ox* tested, incubation with 800 ppm *Covi-ox* was found to yield hepatocytes with a higher percentage viability than control cells (84.4 ± 2.2% compared to 75.5 ± 4.5% using trypan blue exclusion) see Fig 3.4.1a. This concentration was used in further studies using *Covi-ox*.

*7-Ketocholesterol ± 800 ppm Covi-ox*

The effect of 7-ketocholesterol on hepatocyte viability is shown in Fig 3.4.1b. Incubation with 7-ketocholesterol seemed to bring about a decrease in cell viability which was significant (p<0.05) at 2000 ppm when trypan blue exclusion was used to determine viability. When LDH leakage was used as a marker of viability, a significant decrease
Fig 3.4.1 showing the effect of (a) Covi-ox, (b) +/- 7-ketocholesterol or (c) +/- cholestanetriol on hepatocyte viability following incubation for 3 hours. *indicates a significant (p<0.05) difference to control **indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox. For all values n=3 errors shown are SEM.
(p<0.05) in viability was seen when cells were incubated with 7-ketocholesterol (1200 ppm)

Co-incubation with 800 ppm Covi-ox seemed to increase cell viability, significantly at 100 ppm 7-ketocholesterol (p<0.05) (Fig 3 4 1b) when using trypan blue exclusion and at all concentrations using LDH leakage.

**Cholestanetriol ± 800 ppm Covi-ox**

Cholestanetriol was found to have no significant effect on cell viability using either trypan blue exclusion or LDH leakage to determine viability (Fig 3 4 1c). An unexpected result is seen in Fig 3 4 1c. Coincubation with 800 ppm Covi-ox was found to adversely affect cellular viability at 100 ppm cholestanetriol. The viability, as determined using trypan blue exclusion, was found to be 76.2 ± 9.4% when cells were incubated with 100 ppm cholestanetriol and 48.1 ± 2.9% when coincubated with 800 ppm Covi-ox (p<0.05). When using LDH leakage to determine viability, no significant difference in viability was found following coincubation with 800 ppm Covi-ox.

**3.4.2 ATP, Gluconeogenesis and Protein Synthesis**

**ATP**

Covi-ox alone had no significant effect on ATP content. Incubation of hepatocytes with 100 and 1200 ppm 7-ketocholesterol had no effect on ATP content but coincubation with 800 ppm Covi-ox did significantly (p<0.05) increase the cellular ATP level. Although a large variation was seen in ATP content, as denoted by the large error values, incubation with 100 and 1200 ppm cholestanetriol in the presence and absence of 800 ppm Covi-ox caused a significant (p<0.05) increase in ATP content.
Table 3.4 2a The effect Covi-ox (0 to 1200 ppm) and of 7-ketocholesterol and cholestanetriol ± 800 ppm Covi-ox on ATP levels in hepatocyte suspension culture

<table>
<thead>
<tr>
<th></th>
<th>ATP content, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.6 ± 0.14, n = 3</td>
</tr>
<tr>
<td>Covi-ox</td>
<td></td>
</tr>
<tr>
<td>100 ppm</td>
<td>1.1 ± 0.5, n = 3</td>
</tr>
<tr>
<td>800 ppm</td>
<td>1.1 ± 0.2, n = 3</td>
</tr>
<tr>
<td>1200 ppm</td>
<td>1.7 ± 0.48, n = 3</td>
</tr>
<tr>
<td>7-ketocholesterol</td>
<td></td>
</tr>
<tr>
<td>100 ppm</td>
<td>1.2 ± 0.24, n = 4</td>
</tr>
<tr>
<td>1200 ppm</td>
<td>1.65 ± 0.46, n = 4</td>
</tr>
<tr>
<td>+ 800ppm Covi-ox</td>
<td></td>
</tr>
<tr>
<td>100 ppm</td>
<td>2.25 ± 0.35*, n = 3</td>
</tr>
<tr>
<td>1200 ppm</td>
<td>3.35 ± 0.11*, n = 3</td>
</tr>
<tr>
<td>Cholestanetriol</td>
<td></td>
</tr>
<tr>
<td>100 ppm</td>
<td>2.88 ± 1.4*, n = 3</td>
</tr>
<tr>
<td>1200 ppm</td>
<td>2.4 ± 0.9*, n = 3</td>
</tr>
<tr>
<td>+ 800ppm Covi-ox</td>
<td></td>
</tr>
<tr>
<td>100 ppm</td>
<td>3.1 ± 0.3*, n = 3</td>
</tr>
<tr>
<td>1200 ppm</td>
<td>4.35 ± 0.5*, n = 3</td>
</tr>
</tbody>
</table>

*indicates a significant (p<0.05) difference to control, errors shown are SEM

Glucose Production

At concentrations of 800 and 1200 ppm, Covi-ox was found to significantly (p<0.05) increase glucose production in hepatocytes in suspension culture, Fig 3.4.2a(a), although the increase did not appear to be dependent on Covi-ox concentration (0.112 mg glucose/mg protein was produced in the presence of 800 ppm Covi-ox while 0.074 mg glucose/mg protein was produced in the presence of 1200 ppm Covi-ox). Exposure of
hepatocytes in suspension culture to 100 ppm 7-ketocholesterol was seen to lead to a significant \((p<0.01)\) decrease in glucose production, while exposure to 1200 ppm had no significant effect, Fig 3 4 2a(b). Coincubation with 800 ppm Covi-ox significantly \((p<0.01)\) increased glucose production in both cases (Fig 3 4 2a(b)). Fig 3 4 2a(c) shows the effect of cholestanetriol \(\pm\) 800 ppm Covi-ox on glucose production. Incubation of hepatocytes with cholestanetriol alone had no significant effect on glucose production. Coincubation with 800 ppm Covi-ox was found to significantly \((p<0.05)\) increase glucose production, activity being 3-4 times higher (0.138-0.145 mg glucose/\(10^5\) cells) than in the absence of Covi-ox (0.03-0.04 mg glucose/\(10^5\) cells).
Fig. 3.4.2a. The Effect of Covi-ox +/- Oxysterols on Glucose Production

Fig. 3.4.2a showing the effect of (a) Covi-ox, (b) +/- 7-ketocholesterol or (c) +/- cholestanetriol on hepatocyte viability following incubation for 3 hours. *indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) to the value obtained in the absence of Covi-ox. For all values n=3, errors shown are SEM.
Protein Synthesis

The effect of Covi-ox on protein synthesis is shown in Fig 3 4 2b(a) After an initial increase (p<0.05) at 100 ppm Covi-ox, increasing concentrations of Covi-ox led to decreasing protein synthesis, significant (p<0.05) at 1200 ppm. Both 7-ketocholesterol and cholestanetriol were found to exert a similar effect on protein synthesis, Figs 3 4 2b (b) and (c). Exposure of hepatocytes to 100 ppm of either oxysterol led to a significant (p<0.05) increase in protein synthesis whereas when cells were incubated with 1200 ppm oxysterol no effect was seen. Coincubation with 800 ppm Covi-ox was found to have a more pronounced effect on the cells exposed to cholestanetriol. Although Covi-ox induced a decrease in protein synthesis in both cases, this decrease was significant (p<0.01) in cells exposed to cholestanetriol (Fig 3 4 2b(c)).
Fig. 3.4.2b The Effect of Covi-ox +/- Oxysterols on Protein Synthesis.

(a) The Effect of Covi-ox on Protein Synthesis

![Graph showing the effect of Covi-ox on protein synthesis.](image)

(b) The Effect of 7-Ketocholesterol 800 ppm Covi-ox on Protein Synthesis

![Graph showing the effect of 7-ketocholesterol on protein synthesis.](image)

(c) The Effect of Cholestanetriol ± 800 ppm Covi-ox on Protein Synthesis

![Graph showing the effect of cholestanetriol on protein synthesis.](image)

Fig 3.4.2b showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) or +/- cholestanetriol (c) on protein synthesis as measured by the incorporation of L-2H leucine. For all values n=3, errors shown are SEM. *indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox.

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3 4 3 Antioxidant Enzyme Levels

SOD Activity

Incubation with increasing concentrations of Covii-ox did not significantly affect SOD levels (Fig 3 4 3 a (a)) 7-ketocholesterol had no effect on SOD at concentrations up to 800 ppm while at higher concentrations, SOD activity increased significantly (p<0 05, Fig 3 4 3 a(b)) There was an approximate 2.5 fold increase in SOD activity in cells treated with 2000 ppm 7-ketocholesterol (4 U/mg protein) compared with control cells (1.7 U/mg protein) Inclusion of Covii-ox with 7-ketocholesterol (400, 1200 and 2000 ppm) significantly reduced SOD activity (p<0 05) (Fig 3 4 3 a(b)) Cholestanetriol had no effect on SOD activity at concentrations at or below 1200 ppm (Fig 3 4 3 a(c)) However, SOD activity was increased 1.4 fold (p<0 05) relative to control cells on exposure of liver cells to 2000 ppm cholestanetriol (2.38 U/mg) (Fig 3 4 3 a(c)) Inclusion of Covii-ox in the culture medium significantly reduced SOD activity relative to corresponding cholestanetriol-treated cells (Fig 3 4 3 a(c))

Catalase Activity

The effect of Covii-ox (0-2000 ppm) on catalase activity is shown in Fig 3 4 3 b(a) Activity was decreased significantly in the presence of 1200 and 2000 ppm Covii-ox from a control level of 64.9 Bergmeyer U/mg protein to 16.7 (p<0.01) and 33.7 (p<0.05) Bergmeyer U/mg protein respectively The apparent increase in catalase activity upon exposing cells to 2000 ppm Covii-ox relative to 1200 ppm was not significant Catalase activity in hepatocytes was unchanged in cells exposed to concentrations of 7-ketocholesterol lower than 1200 ppm but increased at higher concentrations A significant increase (p<0.05) was observed in cells exposed to 7-ketocholesterol (2000 ppm) (Fig
Fig. 3.4.3a The Effect of Covi-ox +/-Oxysterols on SOD Activity

Fig. 3.4.3a showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on SOD activity. For all values n=3, errors shown are SEM. * indicates a significant (p<0.05) difference to control, ** indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox.
**Fig. 3.4.3b. The Effect of Covi-ox +/- Oxysterols on Catalase Activity.**

**a. The Effect of Covi-ox on Catalase Activity**

![Graph showing the effect of Covi-ox on catalase activity.](image)

**b. The Effect of Covi-ox on Catalase Activity in 7-Ketocholesterol Treated Cells**

![Graph showing the effect of Covi-ox on catalase activity in 7-ketocholesterol treated cells.](image)

**c. The Effect of Covi-ox on Catalase Activity in Cholestanetriol Treated Cells**

![Graph showing the effect of Covi-ox on catalase activity in cholestanetriol treated cells.](image)

Fig. 3.4.3b showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on Catalase activity. For all values n=3, errors shown are SEM. * Indicates a significant (p<0.05) difference to control, ** indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox.
Covi-ox maintained catalase activity at near control levels when incubated with all concentrations of 7-ketocholesterol. Only in cells exposed to 1200 and 2000 ppm 7-ketocholesterol did coincubation with Covi-ox induce a significant decrease (p< 0.01) in catalase activity (Fig 3 4 3 b(b)). Incubations with up to 800 ppm cholestanetriol caused a dose-dependent increase (p<0.05) in catalase activity (Fig 3 4 3 b(c)), reaching 120 Bergmeyer U/mg protein) but at concentrations of cholestanetriol above 800 ppm catalase activity decreased to control level. The trend in catalase activity when cells were incubated with cholestanetriol was also seen when cells were incubated with cholestanetriol in the presence of Covi-ox but reached a peak at 400 ppm. However, this activity was significantly lower than that observed in cells incubated with cholestanetriol alone from 400 - 2000 ppm (Fig 3 4 3 b(c)).

**GPx Activity**

Figure 3 4 3 c(a) shows the effect of Covi-ox (0 - 2000 ppm) on GPx activity. Despite variable GPx activity in cells exposed to Covi-ox (0 – 1200 ppm) there was a significant 2-fold increase (p<0.05) in GPx activity (0.8 U/mg protein) at the highest concentration studied (2000 ppm) relative to control cells (0.4 U/mg protein).

7-Ketocholesterol had no significant effect on GPx activity (Fig 3 4 3 c(b)). Coincubation with Covi-ox resulted in GPx activity that was lower than in the absence of Covi-ox, but due to large variation in GPx activity, the decrease did not attain statistical significance (Fig 3 4 3 c(b)). Cholestanetriol similarly produced variable effects on GPx activity (Fig 3 4 3 c(c)). Inclusion of Covi-ox (800 ppm) in medium containing cholestanetriol (100 – 2000 ppm) maintained GPx activity at a level significantly higher (p<0.05) than control.
Fig. 3.4.3c The Effect of Covi-ox +/- Oxysterols on GPx Activity

a. The Effect of Covi-ox on GPx Activity

b. The Effect of Covi-ox on GPx Activity in 7-Ketocholesterol Treated Cells

c. The Effect of Covi-ox on GPx Activity in Cholestanetriol Treated Cells

Fig. 3.4.3c showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on GPx activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox.
level In the presence of 2000 ppm cholestanetriol, Cov-ox led to a significant reduction (p<0.05) in GPx activity from 0.83 to 0.59 U/mg protein (Fig 3.4.3(c)).

3.4.4 Lipid Peroxidation

Neither Cov-ox (0 - 2000 ppm), 7-ketocholesterol or cholestanetriol (400 - 2000 ppm) alone affected lipid peroxidation as measured by TBARS (Fig 3.4.4). However, TBARS were significantly (p<0.05) lower in both 7-ketocholesterol (400, 800, 1200 ppm) and cholestanetriol (100 and 400 ppm) - treated cells in the presence of Cov-ox than in its absence (Fig 3.4.4(b),(c)). Cov-ox had negligible effects at higher concentrations of oxysterol.
Fig. 3.4.4 showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on lipid peroxidation as measured by TBARS. For all values $n=3$, errors shown are SEM. **Indicates a significant ($p<0.05$) difference to the value obtained in the absence of Covi-ox.
3.5 Discussion

Hepatocyte suspension cultures were chosen as a model system to study the protective effect of a tocopherol mixture (Covi-ox) against the effects of two commonly occurring dietary oxysterols in the concentration range 0 – 2000 ppm. The manufacturers of Covi-ox, Henkel Ltd, recommend including 100 to 2000 ppm Covi-ox in foodstuffs and these concentrations were used in the study. Covi-ox alone was found to have no significant effect on cellular viability (Fig 3.4.1(a)) although co-incubation with 7-ketocholesterol led to an increase (p<0.05) in the viability of hepatocytes compared to incubation with 7-ketocholesterol alone (Fig 3.4.1(b)). 7-Ketocholesterol alone was found to significantly decrease hepatocyte viability (p<0.05) at higher concentrations (1200 and 2000 ppm, Fig 3.4.1(b)). Covi-ox is seen therefore to exert a protective effect, combating the damage induced by exposure to high concentrations of 7-ketocholesterol. The opposite effect was seen when Covi-ox was co-incubated with 100 ppm cholestanetriol. At this concentration of cholestanetriol, co-incubation with 800 ppm Covi-ox was found to significantly decrease (p<0.05) cellular viability compared to incubation with cholestanetriol alone (Fig 3.4.1(c)). Covi-ox was found to have no such effect at 1200 ppm cholestanetriol or when viability was assessed using LDH leakage. In these cases no effect was observed. Cholestanetriol alone was found to have no significant effect on cellular viability (Fig 3.4.1(c)). This result contradicts much of what has been published in the literature. Biswas et al. (1964) found that of 103 sterols tested, cholestanetriol was the most cytotoxic to chick heart cultures and Peng et al. (1979) found that 10 μg/ml cholestanetriol and 50 μg/ml 7-ketocholesterol induced necrosis in 25% of smooth aortic muscle cells of New Zealand White Rabbits over a 24 hour period. However, Ohtani et al.
(1996) studied the effects of 7-ketocholesterol on rat hepatocyte viability over 24 hours and found no effect on viability when assessing LDH release. The study also included the determination of viability using the neutral red assay. The neutral red assay involves incubation of the cells with a neutral red dye and then quantification of the dye taken up by the lysosomes. It does not measure membrane integrity. Using this method, 50% of cells were found to be non-viable. This would indicate that 7-ketocholesterol may indeed be cytotoxic but that this cytotoxicity does not involve a breach of the cellular membrane. This result highlights the limitations of both enzyme release and trypan blue staining as methods for determining cellular viability. In the same study, ladder-like fragmentation of DNA was observed indicating a possible role for 7-ketocholesterol in the stimulation of apoptosis (Ohtani et al., 1996). This would explain a decrease in viability with no concurrent release of cellular contents. A further explanation for the lack of cytotoxic effects at all concentrations of two compounds that have been widely reported to be cytotoxic (Biswas et al., 1964, Imai et al., 1976, 1978, Bing et al., 1979, Peng et al., 1979, Chan and Chan, 1980, Guardiola et al., 1996, Ohtani et al., 1996) is that the studies reported were of a much longer duration, at least 24 hours compared to the three hour incubations used in the present study. Therefore, further study using a more long-term culture system and alternate methods of determining viability would seem advisable. The determination of ATP content did not seem to aid in the determination of cellular viability. Although satisfactory for the determination of ATP content in freshly isolated rat hepatocytes, a large variation was seen in the data gathered for ATP content of rat hepatocytes following a three hour incubation with COPs in the presence and absence of Covi-ox. The large error values obtained, up to 64% of the ATP content, indicate a lack

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of reproducibility in the procedure. However, a significant increase in ATP content was seen in hepatocytes incubated with 100 and 1200 ppm cholestanetriol in the presence and absence of 800 ppm Covi-ox and with 100 and 1200 ppm 7-ketocholesterol in the presence of 800 ppm Covi-ox. 100 and 1200 ppm 7-ketocholesterol and 100–1200 ppm Covi-ox alone had no significant effect on ATP content (Table 3 4 2a).

The effects of exposure of hepatocytes to Covi-ox on glucose production are more striking. At concentrations at and greater than 800 ppm Covi-ox, glucose production was significantly (p<0.05) increased (Fig 3 4 2a(a)). Co-incubation of Covi-ox (800 ppm) with both 7-ketocholesterol and cholestanetriol increased glucose production (Figs 3 4 2a(b) and 3 4 2a(c)). The production of glucose is a liver specific function and therefore indicates a role for the inclusion of a tocopherol supplement in hepatocyte culture medium.

Both 7-ketocholesterol and cholestanetriol induced a significant (p<0.01) increase in protein synthesis when incubated for three hours with hepatocytes at a concentration of 100 ppm. Co-incubation with 800 ppm Covi-ox seemed to decrease this, significantly (p<0.05) with 100 ppm cholestanetriol. No significant effect on protein synthesis was observed when 1200 ppm of either oxysterol was incubated in the presence or absence of 800 ppm Covi-ox. Covi-ox, after inducing an initial increase in protein synthesis at 100 ppm, caused a dose-dependent decrease in protein synthesis which reached significance (p<0.05) at 1200 ppm. Ohtani et al. (1996) reported that vitamin E significantly (p<0.01) inhibited the incorporation of 7-ketocholesterol into rat hepatocytes. This may explain the decrease in protein synthesis observed when Covi-ox was incubated with 100 ppm cholestanetriol (Fig 3 4 2b(c)). As the protein synthesis assay relies on the uptake of a
labeled precursor from the culture medium, an inhibitory effect of Covi-ox on precursor uptake into hepatocytes would explain the dose dependent decrease in protein synthesis observed in hepatocytes incubated with 0-1200 ppm Covi-ox (Fig 3 4 2(c))

Tocopherol has been found to have antioxidant properties both in vivo and in vitro (Vasavi et al, 1994) but Mak et al, (1996) found that high concentrations of vitamin E (more than 1 g/kg) can have a prooxidant effect. Incubation with up to 2000 ppm Covi-ox did not appear to induce any prooxidative effects and even seemed to slightly reduce lipid peroxidation at the highest concentrations (Fig 3 4 4(a)) GPx activity remained close to control levels except at 2000 ppm Covi-ox where it was seen to increase significantly (p<0.01) while the activities of the antioxidant enzymes SOD and catalase were unaltered or decreased, that of catalase being significantly reduced at Covi-ox concentrations in excess of 1200 ppm (p<0.05). Thus destruction of possible hydroxyl or lipid peroxyl species by GPx activity in cells exposed to high concentrations of Covi-ox in hepatocyte culture medium ensures against prooxidative effects of tocopherols in biological membranes.

The question of whether the cholesterol oxides 7-ketocholesterol and cholestanetrol have themselves oxidative properties has been resolved. Induction of oxidative stress is expected to lead to an increase in the level of activity of the antioxidant defense enzymes and finally to lipid peroxidation if the endogenous defense system is overwhelmed (Halliwell and Gutteridge, 1989). On incubation with increasing concentrations of 7-ketocholesterol, there was a sharp increase in SOD activity above 800 ppm 7-ketocholesterol. SOD is known to be the first line of antioxidant defense dismuting superoxide radicals to hydrogen peroxide and water and therefore such an
increase points to onset of oxidative stress above a threshold concentration of 800 ppm 7-ketocholesterol. As hydrogen peroxide, the end-product of SOD activity, is also causative of oxidative stress, SOD acts with a second enzyme (either catalase or GPx) to remove the hydrogen peroxide formed. Although 7-ketocholesterol was found to have no effect on GPx activity, catalase activity was seen to increase when cells were incubated with greater than 800 ppm 7-ketocholesterol.

On examination of the extent of lipid peroxidation it was found that the increased levels of SOD and catalase were sufficient to protect the cells from oxidation. Lipid peroxidation as measured using the TBARS method was found to remain close to the control level of 1.4±0.1 nmol MDA/mg protein, a value consistent with that reported by Vasavi et al. (1994) who obtained a value of 1.1±0.18 nmol MDA/mg protein as a measure of lipid peroxidation present in control rat hepatocytes.

By contrast with 7-ketocholesterol, a much higher concentration of cholestanetriol (2000 ppm) was required to induce SOD activity in liver cells. The increased SOD activity was accompanied by an increase in GPx activity. Thus we can assume that lipid peroxidation in liver cells was prevented due to the actions of either catalase or GPx activity at lower concentrations of cholestanetriol, and to the combined effects of SOD and GPx at higher concentrations. That lipid peroxidation in hepatocytes was reduced when cholestanetriol and Covi-ox were simultaneously present in the culture medium lends further support to the theory that reactive peroxyl radicals were produced by cholestanetriol at as low a concentration as 800 ppm cholestanetriol.

Inclusion of 800 ppm Covi-ox in culture medium with either cholesterol oxide had a protective effect on the cells with respect to both antioxidant defense enzymes and
lipid peroxidation. The restoration of enzyme levels to control values suggests that the oxidative stress induced by the cholesterol oxides was controlled by the action of Coviox.

3.6 Summary

Suspension cultures of isolated rat hepatocytes were used to investigate whether 7-ketocholesterol and cholestanetriol exert oxidative stress in cells as manifested by lipid peroxidation and the induction of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase. The oxysterols were found to increase the levels of both superoxide dismutase and catalase and to have variable effects on glutathione peroxidase activity. No increase in lipid peroxidation was observed leading to the conclusion that the endogenous antioxidant defense system is capable of protecting against any oxidative stress that might otherwise be exerted by 7-ketocholesterol or cholestanetriol. Coviox, a natural tocopherol blend, reduced the effects of both oxysterols on the antioxidant enzymes examined. The data strengthens the relevance of standardized and adequate supplementation of hepatocyte tissue culture media with tocopherols to improve the antioxidative status of cultured cells that are oxidatively challenged.
CHAPTER 4

An Investigation of the Antioxidant Properties of CLA in
Hepatocyte Suspension Cultures
4.1 Introduction

Conjugated Linoleic Acid (CLA) is a mixture of isomers, both positional and geometric, of linoleic acid. It has two double bonds, which can be in positions 9 and 11, 10 and 12 or 11 and 13. Each of these can be in the cis or trans configuration giving rise to 8 separate isomers. The alternation between single and double bonds, which is found in the molecule, is known as conjugation, hence the name "conjugated linoleic acid."

CLA is normally found as a minor constituent of the lipid fraction of many foods but highest levels are found in ruminant meat and dairy products. CLA can be produced by ruminant bacteria via the biohydrogenation of linoleic acid. The ruminant bacterium Butyrivibrio fibrisolvens has been found to convert linoleic acid to stearic acid via a CLA intermediate (Kepler et al., 1966) and meat from ruminants has been found to contain 3-6 mg CLA/g fat whereas meat from non-ruminants contains less than 1 mg CLA/g fat (Chin et al., 1992). CLA can however be produced in other ways as cooking has been seen to increase the CLA content of meat (Ha et al., 1989).

Of the isomers of CLA, biological activity is linked with the cis-9, trans-11 isomer only. This isomer also seems to be preferentially formed by rumen bacteria during the biohydrogenation of linoleic acid (Hughes et al., 1982). Linoleic acid is an 18 carbon chain essential polyunsaturated fatty acid. It also has two double bonds although they are in positions 9 and 12 and are both in the cis configuration. It is predominantly found in vegetable oils.

Conflicting reports have been published concerning the fate of CLA following ingestion. Belury and Kempa-Steczko, (1997) fed female SENCAR mice semipurified diets which contained 0%, 0.5%, 1% and 1.5% CLA by weight for 6 weeks. Such a
feeding regime was found to decrease body weight but increase the total extractable lipid content of liver. When the separate lipid fractions were examined, CLA was found to have been taken up into the neutral lipid fraction, altering the normal lipid composition via a decrease in arachidonate and an increase in oleate levels. CLA was also found to be taken up into the phospholipid fraction, this time at the expense of linoleate (Belury et al., 1997). A study carried out by Ip et al. in 1996 however reported the predominant incorporation of CLA in neutral lipids of mammary tissue and only found a minimal increase in the CLA content in the phospholipid fraction. Moreover, the fatty acid content of these fractions was reported to have remained unchanged by the dietary supplementation of CLA. The minimal increase in phospholipid CLA seemed to be due to the incorporation of the cis-9, trans-11 isomer of CLA only whereas all isomers of CLA were incorporated into the neutral lipid fraction (Ha et al., 1990 and Ip et al., 1991). These studies, carried out on both liver and mammary tissue, reported no marked change in the oleic or linoleic acid composition of the phospholipid fraction.

Initial interest in CLA was linked to the discovery of an anti cancer effect. CLA has been found to have a protective effect in many animal cancer models. The methylN-trosurea (MNU) model was used to study the efficacy of CLA in protecting against mammary cancer in developing rats (Ip et al., 1995). The diet of early post-weaning and adolescent rats was supplemented with CLA prior to MNU administration. The total tumour yield was reduced by over 40%. When CLA was administered after MNU treatment constant feeding of CLA was necessary to induce a significant reduction in tumour yield. MNU does not require activation thus ruling out the role of CLA in carcinogen activation.
Ha et al topically applied CLA at 7 days, 3 days and 5 minutes prior to 7,12-dimethyl-benz[a]anthracene (DMBA) treatment of mice to initiate two stage mouse epidermal cancer. Control mice were painted with linoleic acid. Following the administration of 12-o-tetradecanoylphorbol-13-acetate as a promoter, CLA was found to reduce the incidence of papillomas by 50% (Ha et al, 1987). CLA has also been found to reduce the incidence of forestomach tumours in mice induced by benzo[a]pyrene by approximately 50% (Ha et al, 1990).

A study published recently by Belury et al (1997) outlining the first possible negative effect of CLA, postulated that CLA may act as a peroxisome proliferator. These compounds, although not chemically linked, all have a hydrophobic region and an acidic group and are responsible for hepatic lipid accumulation, a decrease in body weight and possibly for altering the fatty acid composition of liver. As most peroxisome proliferators are associated with hepatocarcinogenesis, a study was carried out where female SENCAR mice were fed diets of 0-1.5% CLA for 6 weeks. Levels of acyl CoA oxidase (ACO), fatty acid binding protein (FABP) and cytochrome P450 4A1 mRNA and protein were measured. The levels of both mRNA and protein were found to be increased, with the 1% diet of CLA being most effective. Induction of these proteins indicate that CLA may indeed be a peroxisomal proliferator. To investigate a possible link between peroxisomal proliferators and hepatocarcinogenesis, the level of ornithine decarboxylase (ODC), a measure of cell proliferation and tumour promotion, was determined. The level of ODC was increased 10-fold through supplementation of the diet with 1% or 1.5% CLA. This led the authors to conclude that the chemoprotective effect of CLA observed in
extrahepatic tissues may occur at the expense of enhanced hepatocarcinogenesis (Belury et al., 1997).

CLA has also been linked to an antioxidant function. Panza et al. (1985) found that CLA inhibited peroxide formation by 90% when used in a ratio of 1:1000 CLA:linoleic acid. Ip et al. (1991) studied the antioxidant effect of CLA in mammary and liver tissue using the thiobarbituric acid reactive substances (TBARS) test. A significant decrease in TBARS was found in mammary tissue although this was not dose-dependent. The same reduction was found for all doses from 0.25 to 1.5% CLA fed to rats over a 1 month period. Liver lipid peroxidation, as measured by TBARS, however, was not affected by CLA supplementation. Vitamin E (0.05%), which was also examined, was found to function as an antioxidant in both mammary and liver tissue (Ip et al., 1991).

In this study, the characterised hepatocyte model was used to study the in vitro interactions of CLA and rat hepatocytes. The aims of the study were:

- To examine the effects of CLA on hepatocyte viability and liver specific function
- To examine the uptake of CLA into lipid fractions
- To assess the effect of CLA on cholesterol synthesis as determined by HMG CoA reductase activity
- To investigate the purported antioxidant properties of CLA in liver by examining the effect of CLA on the antioxidant enzymes SOD, catalase and GPx and on lipid peroxidation as measured by TBARS.
4.2 Reagents

Conjugated Linoleic Acid (a synthetic mixture of isomers) was purchased from Nu-Check Prep. Water’s silica Sep-Pak cartridges were purchased from AGB Scientific. Whatman no 1 filter paper was purchased from Lennox Laboratories Ltd. Hank’s Balanced Salt Solution, collagenase from *Clostridium histolyticum* type 1A, Ham’s F12 nutrient medium, Percoll, trypan blue solution, 7-ketocholesterol, cholestanetriol, hydrogen peroxide, thiobarbituric acid, trichloroacetic acid, NADH, pyruvic acid, BSA solution, DNA standard, bisbenzimidazol, ATP standards, glucose, glucose-6-phosphate dehydrogenase, hexokinase, glucose kit, imidazole, glucose-6-phosphate, NADPH and 3-hydroxy-3-methylglutaryl-CoA were purchased from Sigma London Ltd. Ransod and Ransel kits for the determination of SOD and GPx activity were purchased from Randox Laboratories Ltd, Armagh. [3H] mevalonolactone (33 Ci/mmol), 3-hydroxy-3-methyl[3-14C]glutaryl-CoA (45 6 mCi/mmol), L-[4,5-3H]leucine (148 Ci/mmol, distribution of label is as follows: 86.7% at the 5-H position and 13.3% at the 4-H position) and NCS II tissue solubiliser were purchased from Amersham, Canada Ltd. Ecolite scintillation fluid was purchased from ICN Biomedicals, California. All other reagents were purchased from BDH, Poole and Riedel de Haen, Irl.

*Animals*

The animals used were male Sprague Dawley rats, fed standard rat chow.
4.3 Methods

4.3.1 Incubations

A 2000 ppm solution of CLA was prepared in ethanol and aliquots which would give concentrations of 5, 10 and 20 ppm CLA during incubation were transferred to 6 well plates and the ethanol allowed to evaporate. To some plates 0 to 2000 ppm 7-ketocholesterol or cholesstanetriol were also added as described in chapter 3. Cells were incubated in these plates in Ham's F12 nutrient medium for 3 hours at 37° C, 5% CO₂ and >80% relative humidity.

4.3.2 Viability

Viability of hepatocytes following incubation with CLA ± oxysterol was assessed using both trypan blue exclusion and LDH leakage as described in chapter 2.

4.3.3 CLA Uptake into Liver Lipid Fractions

*Lipid Extraction (Folch et al 1957)*

Following incubation with and without CLA and with and without 7-ketocholesterol or cholesstanetriol, hepatocyte cultures were centrifuged at 800 rpm and the supernatant discarded. 20 ml of a 2:1 chloroform methanol solution was added and the solution sonicated. The resulting solution was filtered through Whatman no 1 filter paper. 5 ml distilled water was added and this was centrifuged at 1000 rpm for 20 minutes. The upper aqueous phase was discarded and the lower organic phase, containing the lipid fractions, was evaporated to dryness. The lipids were redissolved in 1 ml chloroform in preparation for the separation of the lipid fractions.
Separation of Lipid Fractions (Hamilton and Comai, 1988)

The extracted lipids were injected onto the top of a Water’s Sep-Pak silica cartridge and the neutral lipids were eluted with 20 ml chloroform at 25 ml per minute. Following this, 10 ml of a 98:2 chloroform:methanol solution was passed through the cartridge to elute the monoglycerides. Finally, the phospholipids were eluted with 30 ml methanol. These fractions were dried under nitrogen, resuspended in 1 ml chloroform and kept at -80°C under nitrogen until analysis.

Fatty Acid Analysis using GC (Chin et al, 1992)

Fatty acid methyl esters (FAME) of neutral and phospholipid fractions were prepared using acid-catalysed methanolation as described by Stanton et al (1997). The lipid sample was evaporated to dryness under nitrogen. 6 ml of 4% methanolic HCl was added and the sample was heated at 60°C for 1 hour, with shaking. When the sample had cooled, 5 ml of a saturated aqueous solution of sodium chloride, saturated with n-hexane, was added. 1 ml n-hexane was added and the solution centrifuged at 1500 rpm for 3 minutes. The organic (top) layer was removed, added to 1 ml water and the washing step was repeated. 5 mg sodium sulphate was added to the resulting organic fraction to remove any remaining water. This was centrifuged at 1500 rpm for 3 minutes and the supernatant removed for analysis. CLA was quantified by gas liquid chromatography using a Varian 3500 GLC (Varian, Harbour City, CA, USA) fitted with a flame ionisation detector (FID). The cis-9, trans-11 and trans-10, cis-12 CLA isomers were identified by retention time with reference to a CLA mix, generously provided by the Food Research Institute.
Institute, University of Wisconsin, (Madison, WI, USA) Separation was carried out on a Supelcowax-10 capillary column (Supelco Inc, Bellefonte, PA) (60 m long X 0.32 mm id, 0.25 μm film thickness) using He as a carrier gas at a pressure of 27 psi. The injector temperature was programmed from 80° C to a final temperature of 200° C at a rate of 100° C/min, without an initial delay and held for 20 min. The detector temperature was 250° C. The column temperature was programmed from an initial temperature of 50° C to a final temperature of 220° C, without an initial delay, at a rate of 20° C/min during each analysis. The column was held at the final temperature of 220° C for 60 min. Collected data were recorded and analyzed on a Minichrom PC system (VG Data System, Manchester, UK). The response factors of the individual fatty acids were calculated relative to the area of the C16 0 which was assigned a response of 1 (Lawless et al (in press)). The quantity of each fatty acid was expressed as a percentage of total fatty acids.

4.3.4 ATP, Gluconeogenesis, Protein and Cholesterol Synthesis

These parameters were assessed as described in chapter 2.

4.3.5 The Effect of CLA on Antioxidant Enzymes

After incubation with or without CLA, with or without 7-ketocholesterol or cholestanetanol, the hepatocytes were tested for catalase, SOD and GPX activity as described in chapter 2.
4.3.6 The Effect of CLA on Lipid Peroxidation

Following incubation, hepatocyte solutions were assayed for the presence of TBARS as described in chapter 2.

4.4 Results:

4.4.1 Viability

CLA

The effect of CLA on hepatocyte viability after 3 hours is shown in Fig 4.4.1(a). There seems to be a trend towards a decrease which is more obvious when LDH is used as the method of determining viability. This decrease became significant at 10 and 20 ppm CLA (p<0.05).

7-ketocholesterol ± CLA

Using trypan blue exclusion to determine viability, co-incubation of 7-ketocholesterol treated cells with 20 ppm CLA led to a further decrease in viability when compared to incubation with 7-ketocholesterol alone. This decrease reached significance (p<0.05) at 1200 and 2000 ppm 7-ketocholesterol (Fig 4.4.1(b)). Using LDH to determine viability this trend was not seen and the only significant (p<0.05) difference was seen when 20 ppm CLA was incubated with control cells (Fig 4.4.1(b)).

Cholestanetriol ± 20ppm CLA

A similar pattern was seen for the viability of hepatocytes after co-incubation with cholestanetriol and 20 ppm CLA as for co-incubation with 7-ketocholesterol and 20 ppm CLA. When trypan blue exclusion was used to determine viability, a trend towards a decrease in viability was seen following co-incubation of 20 ppm CLA. This decrease
was significant at 100-2000 ppm cholestanetriol (p<0.05) (Fig 4.4.1(c)). This trend was mirrored when LDH was used as the method of determining viability although the decrease in viability was only found to be significant at 100 ppm cholestanetriol (Fig 4.4.1(c)).
Fig 4.4.1 The effect of CLA +/- Oxysterols on Viability

(a) The Effect of CLA on Viability

(b) The Effect of CLA on Viability in 7-Ketocholesterol Treated Cells

(c) The Effect of CLA on Viability in Cholestanetriol Treated Cells

Fig 4.4.1 showing the effect of (a) CLA (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on hepatocyte viability following a three hour incubation. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
The neutral lipid and phospholipid profiles of control hepatocytes following a 3 h incubation is shown in Figs 4.4.2a and 4.4.2b. Both neutral lipid and phospholipid fractions contained similar amounts of palmitate, linoleate, and \textit{trans}-11-C_{18.1} acid. Phospholipids contained higher amounts of stearate and arachidonic acid and lower amounts of oleic, linoleic, and CLA compared with neutral lipids. The effect of 20 ppm CLA in the presence and absence of 7-ketocholesterol and cholestanetriol on the quantity of each neutral lipid in the neutral lipid fraction is shown in the Table 4.4.2a. No effect was seen on palmitic acid, \textit{trans} C18 1 acid, linoleic acid, \textit{γ}-linolenic acid, and arachidonic acid. Co-incubation of 20 ppm CLA and cholestanetriol led to a decrease in the quantity of stearic and oleic acid. This decrease was significant ($p<0.05$) at 800, 1200, and 2000 ppm cholestanetriol for stearic acid and at 2000 ppm cholestanetriol for oleic acid. Incubation with 20 ppm CLA leads to a significant ($p<0.05$) increase in the quantity of both \textit{cis}-9, \textit{trans}-11, and \textit{trans}-10, \textit{cis}-12 CLA isomers. Co-incubation with 7-ketocholesterol and cholestanetriol had no effect on this except at 2000 ppm 7-ketocholesterol, where the quantity of CLA was found to be decreased to near control values.

The effect of CLA (20 ppm) in the presence and absence of 7-ketocholesterol and cholestanetriol on the quantity of each lipid in the phospholipid fraction is shown in Table 4.4.2b. No effect was seen on palmitic acid and arachidonic acid. CLA seemed to increase the percentage stearic acid found in the phospholipid fraction but this increase did not reach significance. Co-incubation with both 7-ketocholesterol and cholestanetriol seemed to maintain the stearic acid content at levels close to control. The percentage
Stearic acid was found to be significantly (p<0.05) lower in the presence of 20 ppm CLA and 800 ppm cholestanetriol than in the presence of 20 ppm CLA alone. The oleic acid content of phospholipid was significantly (p<0.05) increased in the presence of 20 ppm CLA. Coincubation with 800, 1200 and 2000 ppm cholestanetriol and 7-ketocholesterol reduced oleic acid content to control levels (p<0.05). There was a trend towards an increase in the trans-11-C_{18:1} content in CLA treated cells but it was not found to be significant. Coincubation with 7-ketocholesterol and cholestanetriol maintained trans-11-C_{18:1}. It is apparent that coincubation with 7-ketocholesterol (800 ppm) and cholestanetriol (2000 ppm) significantly reduced trans-11-C_{18:1} relative to incubations in the absence of oxysterol. Linoleic acid content of phospholipids was not significantly affected by 20 ppm CLA. Coincubation with 7-ketocholesterol and cholestanetriol increased linoleic acid content, significantly at 800 and 2000 ppm 7-ketocholesterol and at 800, 1200 and 2000 ppm cholestanetriol, compared with 20 ppm CLA alone. γ-Linoleic acid was not detected in the phospholipid fraction when hepatocytes were incubated with 20 ppm CLA. Coincubation with 7-ketocholesterol and cholestanetriol yielded a measurable quantity of γ-linoleic acid but this was still only approximately 7% of the control value. Neither the cis-9, trans-11 nor the trans-10, cis-12 isomers of CLA were detected in the phospholipid fraction of control hepatocytes. Following incubation with 20 ppm CLA, 0.68 ± 0.09 of total phospholipid was composed of the trans-10, cis-12 isomer of CLA and 0.81 ± 0.07% was composed of the cis-9, trans-11 isomer. Coincubation with 7-ketocholesterol and cholestanetriol significantly (p<0.05) decreased both CLA isomers in the phospholipid fraction.
Fig 4.4.2.a Fatty Acid Profile of the Neutral Lipid Fraction of Hepatocytes

![Graph showing fatty acid profile](image)

Fig 4.4.2a showing the neutral lipid profile of control hepatocytes following a three hour incubation. For all values shown n=3 and errors shown are SEM.

- **C16:0** = palmitic acid
- **C18:0** = stearic acid
- **C18:1** = oleic acid
- **trans11** = trans-11-C18:2
- **C18:2** = linoleic acid
- **C18:3** = γ-linolenic acid
- **CLA a** = the trans-10, cis-12, isomer of CLA
- **CLA b** = the cis-9, trans-11 isomer of CLA
- **C20:4** = arachidonic acid
Fig 4.4.2b Fatty Acid Profile of the Phospholipid Fraction of Hepatocytes

Fig 4.4.2b showing the fatty acid profile of the phospholipid fraction of liver cells following a three hour incubation. For all values n = 3, errors shown are SEM.

C16:0 = palmitic acid
C18:0 = stearic acid
C18:1 = oleic acid
trans11 = trans-11-C18:1
C18:2 = linoleic acid
C18:3 = γ-linoleic acid
CLA a = the trans-10, cis-12 isomer of CLA
CLA b = the cis-9, trans-11 isomer of CLA
C20:4 = arachidonic acid
Table 4.2a The effect of CLA in the presence and absence of 7-ketocholesterol and cholestanetrol on the fatty acid composition of the neutral lipid fraction of rat hepatocytes

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>20ppm CLA</th>
<th>+ 800ppm 7keto</th>
<th>+1200ppm 7keto</th>
<th>+2000ppm 7keto</th>
<th>+800ppm 7keto tritol</th>
<th>+1200ppm 7keto tritol</th>
<th>+2000ppm 7keto tritol</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>25.1±0.2</td>
<td>24.9±1.5</td>
<td>26.4±0.7</td>
<td>25.9±1</td>
<td>28±1   2</td>
<td>26.3±0.9</td>
<td>27.6±0.8</td>
<td>25.6±1.1</td>
</tr>
<tr>
<td>18:0</td>
<td>10.9±0.4</td>
<td>9.2±0.2</td>
<td>9±0.8</td>
<td>9.9±1</td>
<td>8.4±1   3</td>
<td>7.9±0.3*</td>
<td>6.7±0.7*</td>
<td>7.2±0.4*</td>
</tr>
<tr>
<td>18:1</td>
<td>16.9±0.6</td>
<td>15.6±0.8</td>
<td>15.1±0.4</td>
<td>15.2±0.8</td>
<td>15.4±0.7</td>
<td>13.7±0.1</td>
<td>13.8±0.7</td>
<td>11.9±0.8*</td>
</tr>
<tr>
<td>18:1 trans</td>
<td>3.8±0.1</td>
<td>3.8±0.2</td>
<td>3.6±0.04</td>
<td>3.7±0.2</td>
<td>3.8±0.3</td>
<td>3.6±0.05</td>
<td>3.4±0.4</td>
<td>3.7±0.4</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>27.1±1.7</td>
<td>27.6±1.2</td>
<td>28.3±0.2</td>
<td>26.6±1.7</td>
<td>27.6±0.7</td>
<td>27.2±0.6</td>
<td>28.7±1.1</td>
<td>30.7±0.9</td>
</tr>
<tr>
<td>18:3</td>
<td>15.0±2</td>
<td>14±0.3</td>
<td>14±0.1</td>
<td>15±0.1</td>
<td>15±0.1</td>
<td>11±0.04</td>
<td>16±0.2</td>
<td>13±0.04</td>
</tr>
<tr>
<td>18:2 cis 12</td>
<td>1.4±0.4*</td>
<td>3.9±0.6</td>
<td>3.36±0.01</td>
<td>3.32±0.5*</td>
<td>2.2±0.5</td>
<td>3.5±0.2*</td>
<td>4.2±0.3*</td>
<td>4.2±0.3*</td>
</tr>
<tr>
<td>18:2 cis 9</td>
<td>1.7±0.1</td>
<td>4.1±0.4</td>
<td>3.9±0.2</td>
<td>4.2±0.2*</td>
<td>2.3±0.4*</td>
<td>3.4±0.1*</td>
<td>3.4±0.2*</td>
<td>3.2±0.06*</td>
</tr>
<tr>
<td>20:4</td>
<td>9.2±1</td>
<td>9.1±1</td>
<td>10.4±0.5</td>
<td>9.6±0.8</td>
<td>10.3±0.6</td>
<td>10.3±0.8</td>
<td>10.6±1.2</td>
<td>11.9±0.9</td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=3 Values are expressed as percentage total fatty acid. * indicates a significant (p<0.05) difference to control and ** indicates a significant (p<0.05) difference to the value obtained in the presence of 20ppm CLA alone.
Table 4.4.2b The effect of CLA in the presence and absence of 7-ketocholesterol and cholestanetriol on the fatty acid composition of the phospholipid fraction of rat hepatocytes

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Control</th>
<th>20ppm CLA</th>
<th>+800ppm CLA</th>
<th>+1200ppm CLA</th>
<th>+2000ppm CLA</th>
<th>+800ppm 7keto</th>
<th>+1200ppm 7keto</th>
<th>+2000ppm 7keto</th>
<th>7keto triol</th>
<th>1200ppm triol</th>
<th>2000ppm triol</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>24.1±0.2</td>
<td>25.5±1.3</td>
<td>24.9±0.2</td>
<td>25.5±0.6</td>
<td>25.6±0.4</td>
<td>26.7±0.9</td>
<td>24.9±1.2</td>
<td>25.6±0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>17.2±1.8</td>
<td>22.±1.5</td>
<td>19.3±0.05</td>
<td>18.3±0.3</td>
<td>18.9±0.3</td>
<td>16.5±0.7*</td>
<td>18.9±0.2</td>
<td>16.8±1.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>4.2±0.7</td>
<td>6.7±0.6c</td>
<td>4.7±0.2*</td>
<td>4.7±0.14*</td>
<td>4.5±0.2*</td>
<td>4.2±0.2*</td>
<td>4.8±0.2*</td>
<td>4.7±0.03*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1 trans</td>
<td>4.9±1.2</td>
<td>6.4±0.8</td>
<td>4.7±0.2*</td>
<td>5.2±0.3</td>
<td>5.5±0.4</td>
<td>4.7±0.3</td>
<td>4.9±0.2</td>
<td>4.8±0.04*</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>11</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>21.7±1</td>
<td>18.1±1.6</td>
<td>22.6±0.2*</td>
<td>21.8±0.7</td>
<td>23.6±0.5*</td>
<td>22.7±0.2*</td>
<td>22.9±0.4*</td>
<td>22.6±0.3*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3</td>
<td>2±0.2</td>
<td>0</td>
<td>14±0.02*</td>
<td>16±0.03*</td>
<td>13±0.02*</td>
<td>1±0.01*</td>
<td>14±0.02*</td>
<td>17±0.02*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 cis 12</td>
<td>0</td>
<td>0.7±0.1c</td>
<td>0.2±0.02*</td>
<td>0.2±0.01*</td>
<td>0.2±0.02*</td>
<td>0.2±0.04*</td>
<td>0.2±0.01*</td>
<td>0.1±0.01*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2 cis 9</td>
<td>0</td>
<td>0.8±0.1c</td>
<td>0.4±0.05*</td>
<td>0.4±0.03*</td>
<td>0.3±0.02*</td>
<td>0.3±0.02*</td>
<td>0.4±0.04c</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4</td>
<td>26±2.4</td>
<td>20.7±1.7</td>
<td>22.9±0.1</td>
<td>23.7±0.3</td>
<td>23.7±0.5</td>
<td>24±0.2</td>
<td>23.7±0.3</td>
<td>25.2±1.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SEM, n=3. Values are expressed as percentage total fatty acid. * indicates a significant (p<0.05) difference to control and ** indicates a significant (p<0.05) difference to the value obtained in the presence of 20ppm CLA alone.
### ATP, Gluconeogenesis, Protein and Cholesterol Synthesis

**ATP Content**

The effect of CLA on the ATP content of hepatocytes is shown in Table 4 4 3a

**Table 4.4 3a The effect of CLA on the ATP content of hepatocytes in the presence and absence of 7-ketocholesterol and cholestanetriol**

<table>
<thead>
<tr>
<th>CLA/oxysterol alone</th>
<th>In the presence of 20 ppm CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>0.6 ± 0.14, n = 3</td>
</tr>
<tr>
<td><strong>CLA</strong></td>
<td></td>
</tr>
<tr>
<td>5ppm</td>
<td>0.53 ± 0.12, n = 3</td>
</tr>
<tr>
<td>10ppm</td>
<td>3.45 ± 2.12, n = 3</td>
</tr>
<tr>
<td>20ppm</td>
<td>0.6 ± 0.06, n = 3</td>
</tr>
<tr>
<td><strong>7Ketocholesterol</strong></td>
<td></td>
</tr>
<tr>
<td>100ppm</td>
<td>1.2 ± 0.24, n = 4</td>
</tr>
<tr>
<td>1200ppm</td>
<td>1.65 ± 0.46, n = 4</td>
</tr>
<tr>
<td><strong>Cholestanetriol</strong></td>
<td></td>
</tr>
<tr>
<td>100ppm</td>
<td>2.88 ± 1.4*, n = 3</td>
</tr>
<tr>
<td>1200ppm</td>
<td>2.4 ± 0.95*, n = 3</td>
</tr>
</tbody>
</table>

Errors shown are SEM, *indicates a significant (p<0.05) difference to control

Although a large variation was seen in ATP content, as denoted by the large error values, incubation with 100 and 1200 ppm cholestanetriol in the presence and absence of 20 ppm
CLA caused a significant (p<0.05) increase in ATP content. Incubation of hepatocytes with 100 and 1200 ppm 7-ketocholesterol had no effect on ATP content but coincubation with 20 ppm CLA significantly (p<0.05) increased the ATP content in cells incubated with 1200 ppm 7-ketocholesterol. Incubation with 0-20 ppm CLA alone had no effect on ATP content.

**Glucose Production**

Incubation of CLA with hepatocytes seemed to induce an initial increase in glucose production although this increase was not significant. However, at 20 ppm CLA a significant (p<0.02) decrease in glucose production was observed (Fig 4.4.3a(a)). CLA was found to have no significant effect on glucose production when co-incubated with 7-ketocholesterol (Fig 4.4.3a(b)). A significant (p<0.05) increase was observed when 20 ppm CLA was co-incubated with 100 ppm cholestanetriol (Fig 4.4.3a(c)).

**Protein Synthesis**

Incubation of hepatocytes with CLA seems to lead to a dose dependent increase in protein synthesis up to a concentration of 10 ppm CLA (p<0.05). At 20 ppm CLA however, a return to levels not significantly different from control was observed (Fig 4.4.3b(a)). 20 ppm CLA was found to lead to a significant (p<0.05) decrease in protein synthesis when co-incubated with 100 ppm 7-ketocholesterol and with 100 ppm...
Fig. 4.4.3a The Effect of CLA +/- Oxysterols on Glucose Production

(a) The Effect of CLA on Glucose Production

(b) The Effect of 7-Ketocholesterol ± 20 ppm CLA on Glucose Production

(c) The Effect of Cholestanetriol ± 20 ppm CLA on Glucose Production

Fig. 4.4.3a showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on glucose production. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
cholestanetriol (Figs 4.4.3b and (c)). No effect was seen when 20 ppm CLA was co-incubated with 1200 ppm 7-ketocholesterol or cholestanetriol.

Cholesterol Synthesis

Fig 4.4.3c shows the effect of 7-ketocholesterol and cholestanetriol with or without 20 ppm CLA on cholesterol synthesis. 7-Ketocholesterol seemed to induce a decrease in HMG CoA reductase activity although this was not significant. Cholestanetriol, however, induced a significant (p<0.05) increase in HMG CoA reductase activity. CLA was found to have no significant effect either alone or during co-incubation with either oxysterol.
Fig. 4.4.3b The Effect Of CLA +/- Oxysterols on Protein Synthesis

(a) The Effect of CLA on Protein Synthesis

(b) The Effect of 7-Ketocholesterol ± 20 ppm CLA on Protein Synthesis

(c) The Effect of Cholestanetriol ± 20 ppm CLA on Protein Synthesis

Fig. 4.4.3b showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on protein synthesis. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
Fig. 4.4.3c The Effect of CLA in the presence and absence of 7-Ketocholesterol and Cholestanetriol on Cholesterol Synthesis

Fig 4.4.3c showing the effect of CLA and oxysterols in the presence and absence of 20 ppm CLA on cholesterol synthesis as measured by the activity of expressed HMG CoA reductase activity. For all values n=4, errors shown are SEM and * indicates a significant (p<0.05) difference to control cells.
The Effect of CLA on Antioxidant Enzymes

**Superoxide dismutase**

The effect of CLA on SOD activity is shown in Fig 4.4.a(a) For all concentrations of CLA examined (5 to 20 ppm) a significant (p<0.05) decrease in SOD activity was seen. The level of SOD activity following incubation with CLA was approximately 20% of control levels. The presence of 7-ketocholesterol did not seem to change the effect of CLA on SOD activity. CLA reduced the level of SOD activity to approximately 20% of control even at 1200 and 2000 ppm 7-ketocholesterol where SOD activity in 7-ketocholesterol treated cells alone was significantly (p<0.05) increased (Fig 4.4.a(b)). A similar pattern was seen for cholestanetriol treated cells. Even at 2000 ppm cholestanetriol which significantly (p<0.05) increased SOD activity in the absence of CLA, SOD activity was reduced to 20% of control levels in the presence of CLA (Fig 4.4.a(c)).

**Catalase**

Incubation with 5 and 10 ppm CLA increased catalase activity in hepatocytes, although the increase was not significant (Fig 4.4.d). However, at 20 ppm CLA, catalase activity was significantly (p<0.05) reduced. When CLA (20 ppm) was incubated with 7-ketocholesterol-treated hepatocytes, a trend towards a reduction in catalase activity was seen when compared to activity in cells treated with 7-ketocholesterol alone (Fig 4.4.e). Catalase activity was significantly decreased (p<0.05) in the presence of 100, 400 and 2000 ppm 7-ketocholesterol. Coincubation of 2000 ppm 7-ketocholesterol and CLA reduced catalase activity to a level similar to catalase activity in control cells. Fig 4.4.f shows the effect of CLA on cholestanetriol-treated cells. In cells incubated with
Fig. 4.4.4a The Effect of CLA +/- Oxysterols on SOD Activity

**Fig. 4.4.4a showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on SOD Activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.**
Fig. 4.4.4b The Effect of CLA +/- Oxysterols on Catalase Activity

Fig. 4.4.4b showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on catalase activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
up to 800 ppm cholestanetriol, a trend towards a decrease in catalase was seen during co-incubation with 20 ppm CLA. However, this decrease was not found to be significant and at concentrations greater than 800 ppm, CLA did not seem to have any effect.

**GPx Activity**

CLA (10 ppm) significantly (p<0.05) decreased GPx activity in rat hepatocytes (Fig 4.4.4 c(a)). At concentrations of CLA above 10 ppm no significant effect on GPx activity was seen. As mentioned in chapter 3, treatment of hepatocytes with 7-ketocholesterol was seen to generate very variable GPx activities. Co-incubation with CLA seemed to reduce that variability and seemed to reduce GPx activity slightly although no significant reduction was seen except at 100 ppm 7-ketocholesterol (p<0.05, Fig 4.4.4 c(b)). No trend in GPx activity was seen in cholestanetriol treated cells. As mentioned in chapter 3, at some concentrations of cholestanetriol (100, 800, 2000 ppm) GPx activity was significantly increased (p<0.05) whereas at others, (400, 1200 ppm) it was not. Again co-incubation with 20 ppm CLA seemed to remove this variability and significantly (p<0.05) reduced GPx activity at 100, 800, 1200 and 2000 ppm cholestanetriol (Fig 4.4.4c(c)).

**4.4.5 The Effect of CLA on Lipid Peroxidation**

CLA was found to have no effect on lipid peroxidation as measured by TBARS when incubated with control cells (Fig 4.4.5(a)). However, a significant increase (p<0.05) in lipid peroxidation was seen in 7-ketocholesterol-treated cells following co-incubation with 20 ppm CLA relative to treatment with 7-ketocholesterol alone (Fig 4.4.5(b)).
Fig. 4.4.4c The Effect of CLA +/- Oxysterols on GPx Activity

For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
similar increase was not seen in cholestanetriol treated cells where lipid peroxidation did not seem to be affected by CLA (Fig 4 4 5(c))
Fig. 4.4.5 The Effect of CLA +/- Oxysterols on Lipid Peroxidation

Fig. 4.4.5 showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on Lipid Peroxidation as measured by TBARS. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.
4.5 Discussion

Hepatocyte suspension cultures were used as a model for short-term studies of the effects of CLA on a number of housekeeping functions of liver cells. The apparent sensitivity of liver cells to CLA as was evident from the decrease in viability occurring in liver cells exposed to increasing concentrations of CLA was not associated with gross impairment of metabolic function as reflected in measurements of ATP, protein or cholesterol synthesis. Protein synthesis was increased upon exposure to CLA at as low a concentration as 10 ppm and both cellular ATP levels and the specific activity of HMG CoA reductase, the rate limiting enzyme of cholesterol biosynthesis, remained unaltered. Despite recent findings that diets containing 1 and 1.5% CLA were associated with hepatic peroxisome proliferation and induction of ornithine decarboxylase (Belury, 1997), no study has yet shown cytotoxic effects of CLA in liver comparable to those observed in cancer cells (DesBordes and Lea, 1995, Shultz et al, 1992, Schonberg and Krokan, 1995).

Since recent studies using hepatocellular models have indicated a suppressive effect of ornithine decarboxylase activity on lipid peroxidation (Ogiso et al, 1997) and in light of the numerous studies showing a correlation between lipid peroxidation and cell damage (Wang et al, 1996), an investigation of the effects of CLA on the oxidative tone of liver seemed warranted. In view of the myriad of beneficial biological effects attributed to CLA observed in vivo and in vitro, it is plausible that protection against lipid peroxidation may be a mechanism by which CLA preserves intact the phospholipid milieu of cell membranes for subsequent eicosanoid metabolism or membrane-mediated signalling events. In this study we have shown that CLA is readily incorporated into both
cell membrane phospholipids as well as neutral lipids. It is noteworthy that short term incubations of hepatocytes with 20 ppm CLA resulted in detectable levels of both CLA isomers in the phospholipid fraction, slightly higher levels of stearate and oleic acids compared with control incubations while lowering levels of oxidation-susceptible linolenic and arachidonic acids. As an antioxidant, its presence in phospholipid membranes would be expected to reduce or at least maintain the amount of TBARS produced in cells during culture. Lipid peroxidation, as measured using the TBARS method, was found to remain close to the control level of 1.4 ± 0.09 nmol MDA/mg protein. This value is consistent with that reported by Vasavi et al. (1994) who obtained a value of 1.1 ± 0.18 nmol MDA/mg protein as a measure of lipid peroxidation present in control rat hepatocytes.

The apparent insensitivity of liver cells to CLA-mediated changes in lipid peroxidation may however be a reflection of the ample antioxidant status of liver cells. Induction of oxidative stress is expected to lead to an increase in the level of activity of the antioxidant defense enzymes and finally to lipid peroxidation if the endogenous defense system is overwhelmed. SOD is known to be the first line of antioxidant defense, dismuting superoxide radicals to hydrogen peroxide and water. In this study, SOD was not increased in cells exposed to CLA indicating an absence of $O_2^-$ species, the major initiator of the oxygen radical cascade that feeds into the lipid peroxidation chain reaction. As hydrogen peroxide, the end product of SOD activity, is also causative of oxidative stress, a second enzyme (either catalase or GPx) is required to remove the hydrogen peroxide formed. The observation that 20 ppm CLA caused a significant decrease in catalase activity, while having no significant effect on GPx activity may be
supportive of a possible quenching effect of CLA on potential reactive oxygen species generated as a result of CLA metabolism in liver cells. It has been previously reported that CLA may act as a substrate for microsomal CYP 4A1 hydroxylase enzymes as well as desaturase enzymes involving cytochrome b5 monooxygenase system in liver (Belury and Kempa-Steczko, 1997). It is plausible that if CLA exerts a direct antioxidant scavenging effect, metabolism of CLA to conjugated 18:3 species may proceed without alteration of the cellular oxidative tone. The observation that the higher levels of SOD, catalase and GPx in hepatocytes challenged with oxysterols were reduced to control levels upon exposure to CLA and that lipid peroxidation remained at a level similar to that of control cells may be viewed as indirect evidence of antioxidant activity of CLA in liver. Although this study has shown an antioxidant enzyme-sparing effect in hepatocytes, it does not exclude possible effects of CLA in maintaining levels of other membrane-bound antioxidants such as α-tocopherol as reported previously (Nicolosi et al., 1997). However, the probability that the oxidative susceptibility of CLA may be influenced by severe oxidative stress must not be discounted in view of the apparent increase in lipid peroxidation when cells were coincubated with 7-ketocholesterol and CLA. Impairment of the cellular defense mechanisms by CLA in 7-ketocholesterol treated cells may mask a prooxidant susceptibility and may be significant in understanding prooxidant effects of CLA seen in several cancer cell lines (Schonberg and Krokan, 1995).

Membrane-bound CLA and its desaturated and/or elongated products are likely to compete with other PUFA for phospholipases, cyclooxygenase and lipoxygenase enzyme families. Although liver parenchymal cells are not the major cell type in liver involved in
eicosanoid synthesis (Kuiper et al, 1998), because of the large volume they occupy they represent a significant fraction of total eicosanoids synthesized by liver. Thus, CLA displacement of phospholipid arachidonate is likely to inhibit eicosanoid synthesis and affect tumorigenesis. In view of findings relating increased rates of glycogenolysis to increased eicosanoid production (Dieter et al, 1987), the decreased rate of gluconeogenesis observed in this study may be a reflection of decreased eicosanoid activity in CLA-treated cells.

The significance of the much higher level of CLA incorporation into neutral lipids of hepatocytes relative to phospholipids may be related to the importance of neutral lipids as a storage form of CLA. Deposition of CLA isomers in neutral lipids of hepatocytes was not at the expense of altered ratios of other major fatty acids. Since the liver is the largest organ in the body, hepatic storage of CLA may be an important determinant underlying the chemoprotective effects of CLA seen in mammary gland and colon. To conclude, the apparent sensitivity of antioxidant enzyme defense systems of liver cells to CLA coupled with the lack of effect of CLA on lipid peroxidation in cells serve as indirect evidence that CLA may act as an antioxidant in normal liver cells.

4.6 Summary

The aim of this study was to test the hypothesis that primary rat hepatocytes were resistant to the prooxidative effects of CLA as measured by the amount of thiobarbituric acid reactive substances (TBARS) formed because of compensatory effects on the antioxidant enzyme defense system. Rat hepatocyte suspension cultures were used to investigate the extent of CLA uptake and its effects on the metabolic function and cellular
oxidative defense system. The decrease in hepatocyte viability as measured by lactate dehydrogenase (LDH) leakage was not associated with gross changes in cellular ATP content, protein synthesis or 3-hydroxy-3-methylglutaryl Coenzyme A reductase, the rate limiting enzyme of cholesterol synthesis. Although CLA was preferentially taken up into neutral lipid fractions of hepatocytes, cellular lipid peroxidation remained unchanged despite significant inhibition of superoxide dismutase (SOD) and catalase. The inhibitory effect of CLA on antioxidant defense enzymes was also observed when cells were exposed to 7-ketocholesterol or cholestanetriol, oxysterols capable of inducing SOD, catalase or glutathione peroxidase (GPx). The apparent sensitivity of the antioxidant defense system of liver cells to CLA coupled with the lack of effect of CLA on lipid peroxidation in cells is indirect evidence that CLA is not a prooxidant in hepatocytes.
CHAPTER 5

Final Discussion and Conclusions
The aim of this project was to validate a cell model and then use this model to study the effects of some food components on the cellular antioxidant defense system. The components studied were 7-ketocholesterol and cholestanetriol, two oxysterols that have been extensively reported to be cytotoxic and atherogenic (Mahfouz et al., 1995, Verhagen et al., 1996, Ohtani et al., 1996, Gelissen et al., 1996), Covi-ox, a commercially available blend of tocopherols – natural compounds that are renowned for their antioxidant properties (Burton et al., 1983, Diplock, 1983, Bonorden and Pariza, 1994, Vasavi et al., 1994), and CLA, a lipid that has received much attention due to its inhibitory effect on the growth mammary and cancer tumours and several cancer cell lines (Ha et al., 1990, Ip et al., 1991, Shultz et al., 1992, Schonberg and Krokan, 1995.) However, whether CLA exerts a beneficial or detrimental effect on other cell types is not clear (Van den Berg et al., 1995, Belury et al., 1997)

In order to achieve the overall aim of this project, it was necessary to choose a suitable cell system. Exposure to these food components is through dietary intake and therefore following absorption in the intestine, they should be transported to the liver, the site of metabolism. Liver cells would therefore be exposed to these food components in vivo and therefore are a natural choice of cell type with which to study the effects of these food components in vitro. However, the metabolism of compounds in vivo is more complex than that in vitro, resulting in more metabolites. For example, metabolism may take place in cells other than the liver. The gastrointestinal system, particularly the bacteria situated there, plays an important role in the metabolism of certain compounds, e.g., the formation of mercapturic acids and the reduction of nitro-group containing compounds (Hoogenboom and Kuiper, 1997). Bioactivation and detoxification of certain
compounds may differ between species, and so it is necessary, initially, to study the effects and metabolism of food components and xenobiotics in cell systems before proceeding to \textit{in vivo} animal systems and finally to \textit{in vitro} human cell models. However, there is still an element of risk when extrapolating this data to humans. This is accounted for by using risk factors of 100 or more. As a model system for primary studies on food components, the liver, as the major site of metabolism, is a suitable cell system. As described in chapter 1, liver tissue is constantly exposed to free radical species \textit{in vivo} making hepatocytes the cell type of choice for \textit{in vitro} studies on the antioxidant defense system.

A two-step collagenase perfusion procedure was used to isolate the cells (described in chapter 2). The type of collagenase employed in such an isolation procedure is critical with regard to both cell yield and viability. Commercial suppliers of collagenase (Sigma, Worthington and Boehringer Mannheim) offer a range of collagenases isolated from various sources and containing different amounts of contaminating enzymes. Queral \textit{et al} (1984) compared three different types of collagenase. These were collagenase type IV from \textit{Clostridium histolyticum}, hepatocyte isolation grade, supplied by Sigma Ltd, St Louis, Mo, collagenase no 100502 isolated from \textit{C. histolyticum} supplied by ICN Pharmaceuticals Inc, Cleveland, Ohio and collagenase/dispase (dispase is a neutral protease) isolated from \textit{Achromobacter tophagus} and \textit{Bacillus polymixa}, supplied by Boehringer Mannheim, Indianapolis. Sprague Dawley rats were used as the donor animals. A non-recirculating perfusion system was used and the livers pre-perfused with a solution of 50 ml Hanks' BSS containing 25 mM sodium bicarbonate, 10 \(\mu\)U/ml insulin, 0.5% (w/v) BSA and 1 mM EGTA. Following this, 100
ml of the same solution, without the EGTA but containing 0.05% (w/v) collagenase, was used to perfuse the liver before it was removed and dispersed in 50 ml of buffer containing collagenase and 2 mM calcium chloride. Dispersal was achieved by combing the liver with a wire dog brush and mincing with scalpel blades. Viability was assessed using trypan blue exclusion. Consistently high viability was obtained using either collagenase/dispase (cells were found to be 85.6 ± 3.7% viable) or collagenase no 100502 (cells were found to be 71.6 ± 12% viable). However, when collagenase type IV from *C. histolyticum* was used, the average viability was only 20.7 ± 11.5%.

Berry et al. (1991) also studied the effect of different types of collagenase on the quality of cell preparations. The results obtained did not agree with those of Queral et al. (1984). Berry tested several collagenases from *C. histolyticum*, which contained varying levels of clostrypain, tryptic and caseinase activities. No correlation was found between the proportions of different enzyme activities and the quality or yield of the cell preparations. In contrast to Queral et al. (1983), the use of collagenase from *A. tophagus* both with and without dispase yielded unsatisfactory hepatocyte preparations. Berry used a one-step perfusion system to isolate hepatocytes from Hooded Wistar rats. The one-step perfusion system relies on the calcium concentration of the perfusing solution being minimal, as this will ensure the hemidesmosomes move apart, a preliminary step in cell isolation. The calcium content of the collagenase from *A. tophagus* was found to be sufficient to raise the calcium content of the perfusing medium by 50 μM. When used with the two-step procedure where the calcium concentration of the collagenase solution is not important, a better preparation was achieved although not as good as that reported by Queral et al. (1983). ECVAM recommended that a standard collagenase be distributed by ECVAM to
researchers in order to ensure quality control and to ensure that results obtained can be compared without reference to any effects a particular collagenase blend may have exerted (Blaauboer et al, 1994). However, since a standard collagenase is not available through ECVAM at present, assessment of different types of collagenase prior to use, as suggested by Berry et al (1991), seems advisable. Prior to the start of this project, a study was carried out in our laboratory to determine the optimum collagenase to use for hepatocyte isolation. The collagenases tested were type IV from *C. histolyticum*, type IA from *C. histolyticum* and a collagenase/dispase from *A. tophagus/B. polymixa*. All were obtained from Sigma Ltd, London. Hepatocytes were isolated as described in chapter 2 and viability was assessed by trypan blue exclusion, also as described in chapter 2. The results obtained in this study are shown in Table 5.1.

Table 5.1 The effect of collagenase on hepatocyte yield and viability

<table>
<thead>
<tr>
<th>Collagenase</th>
<th>Cell Yield, per g wet weight</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase IA</td>
<td>6.62 ± 1.93 x 10^7</td>
<td>71.5 ± 3.8</td>
</tr>
<tr>
<td>Collagenase IV</td>
<td>2.04 ± 1.1 x 10^6</td>
<td>55.0 ± 10.6</td>
</tr>
<tr>
<td>Collagenase/dispase</td>
<td>4.68 ± 1.93 x 10^7</td>
<td>26.9 ± 9.36</td>
</tr>
</tbody>
</table>

This work was carried out by Sinead Miggin in 1994 as part of the requirements for MSc in Biological Science, School of Biological Science, Dublin City University. For all values n=3.
Collagenase type IA was found to yield hepatocyte preparations with both the highest yield and percentage viability, this collagenase was therefore used in the present study.

Following isolation, the physical and biochemical integrity of the cells must be assessed. Both Berry et al. (1991) and ECVAM (Blaauboer et al., 1994) recommended using trypan blue exclusion to determine viability following isolation and LDH release to determine viability during culture. These two methods were therefore used in the current study. Both these methods measure the movement of a substance across the cellular membrane and therefore may be more correctly termed as measurements of membrane integrity rather than of viability, as any damage not affecting the membrane would not be detected. Ohtani et al. (1996) observed no change in LDH leakage in hepatocytes treated with 100 μM 7-ketocholesterol although the neutral red assay indicated that viability had decreased to 50% of control. The observation of ladder-like fragmentation of DNA following incubation of hepatocytes indicated that apoptosis had occurred. Apoptosis, being a controlled method of cell death where intracellular contents are packaged into vesicles before being released and removed by macrophages, means that no breach of the cell membrane occurs and therefore trypan blue exclusion and LDH release will not detect this type of cellular damage. Baur et al. (1975) indicated that trypan blue exclusion and LDH release were insufficient as determinants of cellular integrity as they were unable to detect small alterations to the plasma membrane. They recommended the use of succinate stimulation of mitochondrial respiration and measurement of membrane potential. Succinate is metabolised by liver mitochondria more vigorously than any other fuel. The more intact the cells, as measured by trypan blue exclusion, the lower the rate of succinate oxidation (Berry et al., 1991). It is necessary to know the maximum rate of...
succinate oxidation i.e. the rate of succinate oxidation that would occur if all the cells were damaged. This can be achieved by treating an aliquot of cells with digitonin and measuring the succinate oxidation rate. However, the difficulties in ensuring permeability of all cells without causing damage to mitochondrial membranes means that it is very easy to underestimate the maximal respiration rate and therefore to underestimate the respiration rate in the cell samples being studied (Berry et al., 1991). To monitor succinate oxidation, an O2-electrode is used. The oxygen consumption is measured before and after addition of succinate and the rate of succinate-stimulated respiration is calculated from the difference between the two. The percentage of damaged cells may be calculated by dividing the rate of succinate oxidation in the cell sample by the rate of maximal succinate oxidation (Berry et al., 1991). Although Baur et al. (1975) claimed that succinate oxidation was more sensitive at detecting cellular damage than trypan blue exclusion, Berry et al. (1991) disputes this, claiming that the two methods usually correlate and that succinate oxidation is prone to inaccuracies due to the digitonin permeabilisation. Trypan blue exclusion is also a much quicker and more simple assay to perform.

Baur et al. (1975) measured the chloride potential which, in the perfused rat liver, can be used to determine the membrane potential. To determine the chloride potential hepatocytes were incubated at 37°C for 10 minutes with 2.5 μCi ³H₂O/ml, 0.5 μCi ¹⁴C dextran/ml and 0.5 μCi ³⁶Cl/ml. The cells were then separated from the fluid by centrifugal filtration. Radioactivity in both fractions was measured allowing determination of adherent fluid, cellular volume and intracellular chloride concentration. The membrane potential was then calculated. This is a complex and time-consuming method for the determination of viability. Altman et al. (1993) compared uptake of two...
fluorescent dyes, fluorescein diacetate and propidium iodide with that of trypan blue as methods of determining cell viability. Viable cells can take up fluorescein diacetate and then cleave it to produce fluorescein, a process that yields green fluorescent viable cells. Propidium iodide is a DNA-intercalating dye and is taken up by non-viable cells. Following interaction between the dye and the cellular DNA, orange fluorescent non-viable cells result. Murine hybridoma cells were incubated at 37°C for 6 days and aliquots removed initially and on days 1-6 for determination of viability using both trypan blue exclusion and the fluorescent dyes. There was no difference in viability as measured using the three dyes up to day 2. As the cultures became older (day 3-day 6), trypan blue exclusion was found to overestimate viability. The assays were also carried out in serum-containing media. The serum proteins may have formed complexes with the trypan blue dye which inhibits uptake of the dye, even by non-viable cells (Black and Berenbaum, 1964). Therefore, for initial estimates of cell viability, trypan blue seems to be as reliable as fluorescent dyes.

The retention of biochemical function is also an important measure of viability. Cellular damage may result in loss of the ability to maintain and provide energy for metabolic function and growth (Cook and Mitchell, 1989). Cellular energy can be assessed by determination of total ATP concentration, as in this study, or determination of the energy charge of cells. The energy charge is defined as \[ \frac{[\text{ATP}]}{[\text{ATP}]+0.5[\text{ADP}] + [\text{AMP}]} \]. Kits are available commercially, which allow the determination of total ATP concentration using the luciferin-luciferase system. \[^{31}\text{P NMR}\] is a non-invasive method of determining simultaneously the ATP, ADP and AMP concentrations. A major drawback is the lack of availability of such expensive instrumentation. The ATP, ADP
and AMP pools can be labeled with \(^{32}\)P. This, however, involves extraction, chromatographic separation and measurement of each fraction separately making this a time-consuming procedure (Cook and Mitchell, 1989). A simple, rapid photometric method of determination of total ATP content was developed by Pieczonca and Dehn (1993). The method, as used in this study, couples the ATP-dependent phosphorylation of glucose to glucose-6-phosphate and the subsequent oxidation of glucose-6-phosphate by glucose-6-phosphate dehydrogenase with a concurrent reduction of NADP\(^+\) to NADPH, which can be measured spectrophotometrically at 340 nm. A lack of reproducibility, as indicated by the large error values obtained, was observed when cells coincubated with oxysterols, Cov-ox and CLA were tested for ATP content, see tables 3 4 2a and 4 4 3a. Pieczonca and Dehn (1993) also observed run to run variation but the method was clearly sensitive enough to show a decrease in cellular ATP concentration following incubation of rat and human hepatoma cell lines with potassium cyanide, a known inhibitor of the cytochrome oxidase system and so of oxidative phosphorylation (Pieczonca and Dehn, 1993). Correlation of ATP levels with trypan blue exclusion, as measures of viability have not been found to be universal. A good correlation was found by Kuzmits et al (1986) and by Kangas et al in 1984 (Cook and Mitchell, 1989). A poor correlation (r=0.58) between trypan blue exclusion and ATP levels in Ehrlich ascites was found (Cook and Mitchell, 1989). The quantification of DNA or protein synthesis is another method by which the prevalence of biochemical function can be assessed. For non-dividing, metabolically active cells, the rate of protein synthesis rather than DNA synthesis should be examined (Cook and Mitchell, 1989). Although protein synthesis has been claimed to be a very sensitive marker of cell damage (Berry et al, 1991), protein synthesis...
synthesis was found to overestimate the extent of cellular damage during the first 24 hours of culture but agreed with trypan blue exclusion methods after 48 to 72 hours (Cook and Mitchell, 1989) This method of assessing viability may be applicable to more long-term culture systems than were used in the present study Future work should therefore concentrate on measuring apoptosis and other measurements of cellular viability such as measurement of membrane potential, succinate stimulation of mitochondrial respiration, chloride potential and determination of the energy charge of the cell

Following determination of the structural and biochemical integrity of the isolated hepatocytes, it was necessary to ensure that they retained liver function Liver is the major site of gluconeogenesis and cholesterol synthesis Production of glucose and cholesterol would therefore indicate a retention of major liver functions Gluconeogenesis has been used as a measure of viability as well as an indication of the retention of liver function (Berry et al, 1991) The photometric determination of glucose content following incubation of hepatocytes with precursors such as lactate, pyruvate or glycerol, as was used in this study, is the simplest method for the assessment of gluconeogenesis It is also possible to monitor gluconeogenesis by determining the rate of uptake of $^{14}$C lactate This method may underestimate the rate of gluconeogenesis due to isotopic exchange (Berry et al, 1991) Cholesterol is formed exclusively from acetate The rate-limiting step in this process is the conversion of the intermediate $\beta$-hydroxy- $\beta$-methylglutaryl-CoA (HMG CoA) to mevalonate through the action of the enzyme HMG CoA reductase HMG CoA reductase is an integral glycoprotein of the smooth endoplasmic reticulum and consists of two subunits One subunit is responsible for catalytic function while the other anchors the
enzyme via seven membrane spanning regions which project into the lumen of the ER (Liscum et al., 1985) As this enzyme is rate-limiting in the biosynthesis of cholesterol, the activity of this enzyme can be assessed as a measure of cholesterol synthesis. Originally, determination of the activity of this enzyme involved the preparation of microsomes. Microsome preparation involved cell lysis followed by successive centrifugation steps to remove cellular debris finishing with a 60 min ultracentrifugation step at 4°C. The assay procedure involved exposure of the microsomes to NADP⁺, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and ¹⁴C HMG CoA for 30 mins at 37°C before termination of the reaction through the addition of hydrochloric acid and the addition of ³H mevalonic acid for use as an internal standard. A further 30 min incubation was then necessary to lactonise the mevalonic acid. Mevalonate was separated by either solid phase extraction or thin layer chromatography. Both the mevalonate and the HMG CoA fractions were then counted for radioactivity. This assay was extremely time-consuming. The use of digitonin to permeabilise the cells and allow the determination of the enzyme activity in situ greatly reduced the time taken to complete this assay (Geelen et al., 1991). Although maximal HMG CoA reductase activity occurs during the peak of the dark cycle (Clarke et al., 1984), during this study animals were sacrificed during the light cycle. This was to ensure consistency with all other parameters measured. The effects of 7-ketocholesterol, cholestanetriol, Cov1-ox and CLA on cholesterol synthesis in isolated rat hepatocytes should be further studied, ensuring maximal rates of HMG CoA reductase activity by sacrificing donor animals during the dark cycle.
The effects of four micro-food components on the antioxidant defense system of hepatocytes were determined by assessing the level of lipid peroxidation and measuring the activities of the enzymes superoxide dismutase, glutathione peroxidase and catalase following exposure of the hepatocytes to oxysterols, Cov-ox and CLA. In this study, the TBARS method was used to determine the extent of lipid peroxidation. Small amounts of malondialdehyde are formed during membrane peroxidation. The MDA reacts with thiobarbituric acid to form a coloured product that absorbs at 532 nm. The concentration of MDA in lipid systems is not usually high enough to generate a strong colour and the majority of the colour formed in the TBARS reaction is thought to be generated by degradation products of cyclic- and endoperoxides (Halliwell and Gutteridge, 1989). Other compounds also react with thiobarbituric acid to yield a product that absorbs at 532 nm. These include biliverdin (a bile pigment), some PUFAs and the amino acids glutamine, methionine, homocysteine, proline and arginine. The lack of specificity of this assay means that although it is the simplest and most popular means of determining lipid peroxidation, the results obtained should be treated as an estimate of the extent of lipid peroxidation. During lipid peroxidation, the hydrocarbon gases pentane and ethane are formed through decomposition of peroxides in the presence of transition metal ions. Although these gases are only a minor end-product of lipid peroxidation, their detection in exhaled air by gas chromatography offers a non-invasive technique for the estimation of lipid peroxidation in vivo (Halliwell and Gutteridge, 1989). The exhaled air is passed through an adsorbent at low temperature to adsorb and concentrate the hydrocarbons, which are then removed and assayed by GC. The presence of bacteria on the skin and in the gut, which produce hydrocarbon gases, can introduce errors in this procedure. City air.
is also contaminated with hydrocarbon gases from motor vehicles. A large concentration of isoprene is normally found in exhaled air and this is probably formed during the biosynthesis of cholesterol. Lipid peroxidation may also be monitored by following the decrease in the levels of individual fatty acids. This involves lipid extraction and measurement by HPLC or GC following methylation. This procedure must be carried out under nitrogen to avoid peroxidation of the lipids during isolation and analysis of the fatty acids (Halliwell and Gutteridge, 1989). A more recent method which has been developed for the quantification of lipid peroxidation involves measurement of F2-isoprostanes. These are isomers of prostaglandin F2. They are formed in situ on membrane phospholipids by free radical mediated cyclooxygenase-independent peroxidation of arachidonic acid side chains. A2, D2, E2 and J2-isoprostanes can also be formed (Frei, 1998). The measurement of F2-isoprostanes involves isolation followed by separation using solid phase extraction or thin layer chromatography and finally detection using GCMS.

Direct measurement of SOD activity is possible spectrophotometrically by following the decrease in UV absorbance of O2. An electrolytic cell in which oxygen is reduced to O2 at an electrode can also be used. However, the indirect methods, which follow the rate of inhibition of the reaction between O2 and a detector molecule, are the most frequently used (Halliwell and Gutteridge, 1989). The usual O2- generating system is the xanthine-xanthine oxidase system that was used in this study. Using this method, SOD-like activity could be observed if a component of the test solution inhibited the xanthine oxidase enzyme.
Much research involving liver cells centers around the xenobiotic detoxification function of the liver. Following isolation of hepatocytes, it would therefore be necessary to ensure prevalence of cytochrome P450 function. Cytochrome P450 function can be measured fluorometrically in microsomes by exposing the microsomes to a suitable substrate (e.g., 7-ethoxycoumarin, ethoxy-, pentoxy- and benzylloxyphenoxazones), in the presence of NADPH. The increase in fluorescence, due to the formation of the metabolite resorufin, can be followed. 7-ethoxycoumarin is often used as the substrate but by varying the substrates used, the function of different cytochrome P450s can be studied (Burke et al., 1985). Cytochrome P450 activity was not determined during this study as the detoxification function of the liver was not being examined.

A study of the effects of 7-ketocholesterol, cholestanetrol, Cov-oX and CLA on various housekeeping functions and on the antioxidant defense system was undertaken. An initial determination of the effects of these food components on hepatocyte membrane integrity indicated that at higher concentrations (1200, 2000 ppm), 7-ketocholesterol may have damaged the cellular membrane (Fig. 34 1(b)). Cholestanetrol was found to have no effect on membrane integrity (Fig. 34 1(c)). The cytotoxic effects of these oxysterols have been extensively reported (Mahfouz et al., 1995, Verhagen et al., 1996, Ohtani et al., 1996, Gehssen et al., 1996). Severe cytotoxic effects, as indicated by a breach of membrane integrity, were not observed and so a study into the effects of these oxysterols on the rate of apoptosis in hepatocytes seems warranted. A further study into the effects of these compounds on membrane integrity during more long-term culture would also seem advisable. Cov-oX was seen to have no effect on hepatocyte membrane integrity alone but seemed to protect against the effects caused by exposure to high concentrations.
of 7-ketocholesterol. This could be due to inhibition of uptake of 7-ketocholesterol as Ohtani et al. (1996) reported an inhibition of uptake of 7-ketocholesterol by α-tocopherol. CLA was found not to effect membrane integrity, as measured by trypan blue exclusion, although at higher concentrations, an increase in LDH release was observed (Fig 4.4.1(a)). This inconsistency indicates that further study, again using more long-term culture systems, is warranted. CLA was not found to inhibit the effects of the two oxysterols on membrane integrity (% viability as measured by trypan blue exclusion and LDH release) as did Covi-ox and in fact seemed to enhance it (Figs 4.4.1(b), 4.4.1(c)). Cholestanetriol was not seen to effect membrane integrity alone but a significant decrease in viability, as measured by both LDH release and trypan blue exclusion, was observed following coincubation with 20 ppm CLA.

Neither Covi-ox, CLA nor 7-ketocholesterol was found to affect the energy status of the cell (see Tables 3.4.2(a) and 4.4.3(a)) while cholestanetriol was found to significantly (p<0.05) increase total ATP content. Covi-ox and CLA exerted similar effects in the presence of the oxysterols. Coincubation of 7-ketocholesterol induced an increase in ATP levels similar to that observed when coincubated with CLA whereas the high ATP levels observed in cholestanetriol-treated cells were conserved in the presence of both CLA and Covi-ox. The presence of oxysterols maintained the rate of gluconeogenesis close to control although at 100 ppm 7-ketocholesterol significantly (p<0.05) reduced it (Figs 3.4.2a(b), 3.4.2a(c)). Both CLA and Covi-ox had similar effects on gluconeogenesis. An increase in gluconeogenesis was seen at higher concentrations of both compounds followed by a decrease at the highest concentration examined. The rate of gluconeogenesis was significantly (p<0.05) lower in cells
incubated with 20 ppm CLA than in control cells (Fig 4.4.3a). In the presence of the oxysterols, coincubation with either 800 ppm Covi-ox or 20 ppm CLA caused an increase in gluconeogenesis compared with incubation of hepatocytes with oxysterols alone. This indicates a positive effect of Covi-ox and CLA on the maintenance of liver specific function in hepatocytes exposed to oxysterols. CLA and Covi-ox were found to exert similar effects on protein synthesis also. Exposure of hepatocytes to these two compounds alone resulted in an initial increase in protein synthesis followed by a decrease at the higher concentrations examined (Figs 3.4.2a, 4.4.3a). Both 7-ketocholesterol and cholestanetniol induced a significant (p<0.05) increase in protein synthesis at 100 ppm and a return to control levels at 1200 ppm (Figs 3.4.2a(b), 3.4.2a(c)). Coincubation of cholestanetniol with both Covi-ox and CLA yielded a return to control protein synthesis rates (Figs 3.4.2b, 4.4.3b). CLA was more effective than Covi-ox at causing a return of protein synthesis to control levels in 7-ketocholesterol treated cells (Figs 4.4.3(b), 3.4.2(b)).

To study the effect of the food components on the antioxidant defense system, the activities of the enzymes superoxide dismutase, catalase, and glutathione peroxidase were measured. Both 7-ketocholesterol and cholestanetniol were found to increase the activities of these enzymes. Coincubation with Covi-ox resulted in a trend towards a return of these enzymes to control activities. Coincubation with CLA yields similar results. A major difference in the effects of CLA and of Covi-ox observed is that CLA induced a decrease in SOD activity to approximately 20% of control at all concentrations and in the presence and absence of oxysterols while Covi-ox maintained SOD activity at control levels. Lipid peroxidation was unaffected by 7-ketocholesterol and cholestanetniol indicating that an
increase in the response of the antioxidant defense system compensated for any oxidative stress which may otherwise have been exerted by these compounds. Coincubation of the oxysterols with Cov-i-ox led to a decrease in lipid peroxidation which, when examined in conjunction with the ability of Cov-i-ox to return the antioxidant enzyme activities to close to control level indicates the existence of an antioxidant function. CLA was seen to have no effect on lipid peroxidation alone or in the presence of cholestanetnol. CLA, in the presence of 7-ketocholesterol, seemed to lead to an increase in lipid peroxidation but as this increase was not significantly higher than that found in control cells, a prooxidant effect cannot be assigned to CLA. The return of the activity of the antioxidant enzymes to control levels and below, a similar effect to that observed with Cov-i-ox may be indirect evidence for an antioxidant function for CLA in liver cells. Further study, possibly with more long-term culture systems would seem warranted.

**Conclusions**

Isolated hepatocytes in suspension culture were used to examine the effects of four food components on cellular physical and biochemical integrity and on the antioxidant defense system. 7-ketocholesterol and cholestanetnol, although reported to be highly cytotoxic (Mahfouz *et al*, 1995, Verhagen *et al*, 1996, Ohtami *et al*, 1996, Gelissen *et al*, 1996), were found to have no effect on viability (cholestanetnol) or to effect viability only at higher concentrations (1200 and 2000 ppm 7-ketocholesterol). Cov-i-ox and CLA were found to have a similar effect on all biochemical parameters studied both in the presence and absence of oxysterols. 7-Ketocholesterol and cholestanetnol were found to induce an increase in the activity of some or all of the antioxidant enzymes.
SOD, catalase and GPx indicating the production of radical species. This response of the antioxidant defense system was sufficient to prevent an increase in lipid peroxidation. Covi-ox and CLA induced a decrease in the activity of some or all of the antioxidant enzyme activities during coinoculation with the oxysterols while maintaining lipid peroxidation at control levels or below. This indicates a possible antioxidant function for both CLA and Covi-ox. Suspension cultures of rat hepatocytes isolated by the method recommended by ECVAM were found to be a suitable cell model for the initial study of short-term effects of the micro-food components 7-ketocholesterol, cholestanetriol, Covi-ox and CLA on biochemical function and on the antioxidant defense system. For a more in-depth study, the use of more long-term culture systems would seem advisable.
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APPENDIX
A 1 showing a typical graph recorded during the determination of LDH activity in isolated hepatocytes. The assay used is described in chapter 2. Supernatant indicates LDH activity in the cell medium which has leaked from damaged cells. Cellular pellet indicates LDH activity in intact hepatocytes.
A 2 Protein Standard Curve

A 2 showing a typical protein standard curve as used in the determination of the protein content of isolated hepatocytes. The protein assay used is described in chapter 2.
A 3 Standard Curve for DNA Assay

A 3 showing a typical standard curve used in the determination of the DNA content of isolated hepatocytes. The DNA assay used is described in chapter 2.
A 4 Standard Curve for ATP Assay

A 4 Showing a typical standard curve used in the assay to determine the ATP content of isolated hepatocytes. The ATP assay is described in chapter 2.
A 5 Standard Curve for SOD Assay

A 5 showing a typical standard curve used in the assay to determine the SOD activity in isolated hepatocytes following a three hour incubation at 37 degrees C and 5% CO₂. The assay is described in chapter 2.
A 6 Fatty Acid Content of the Phospholipid Fraction of Hepatocytes

A 6 showing a typical chromatogram obtained following the separation of fatty acids of the phospholipid fraction of isolated hepatocytes. The procedure used is described in chapter 4.