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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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

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Signed _Helen Cantwell_   Date _September 1998_

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Acknowledgements

Abstract

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

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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 Characterisation 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, cholesterogenesis 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, Covi-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 Covi-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 Covi-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

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O'Shea, M , **Cantwell, H.**, Devery, R , Lawless, F and Stanton, C (1998) An *in vitro* investigation of the effects of CLA on cells in culture *Int Dairy J (in press)*

Cantwell, H , Devery, R , O'Shea, M and Stanton, C (1998). Differential effects of conjugated linoleic acid on antioxidant defense enzymes in normal liver cells and cancer cells Abstract for 2nd International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease, Helsinki, Finland June 24-27

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

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Cantwell, H (1997) An *in vitro* investigation of the effects of CLA on cells in culture Poster presented at the International Conference on Functional Foods Designer Foods for the Future Cork, September 30th-October 2nd 1997

Cantwell, H (1998) The effect of a conjugated linoleic acid on superoxide dismutase, catalase and glutathione peroxidase in oxidatively-challenged liver cells Poster presented at the 663rd Meeting of the Biochemical Society, UCG, September 4th 1997

Cantwell, H (1998) Differential effects of conjugated linoleic acid on antioxidant defense enzymes in normal liver cells and cancer cells Poster presented at the 2nd International Conference on Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease, Helsinki, Finland June 24-27, 1998

Abbreviations

ε	Molar extinction coefficient
(β)NAD	β-Nicotinamide adenine dinucleotide
μCi	Micro curies
ACO	Acyl CoA oxidase
ADP	Adenine dinucleotide phosphate
ALS	Amyotrophic lateral sclerosis
AMP	Adenine mononucleotide phosphate
Apo	Apoprotein
ATP	Adenosine 5'-triphosphate
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
BSS	Balanced salt solution
CLA	Conjugated linoleic acid
Co A	Coenzyme A
COPs	Cholesterol oxidation products
CuZnSOD	Copper zinc superoxide dismutase
DEAE-dextran	Diethylaminoethyl-dextran
DiI	3, 3'-Dioctadecylindocarbocyanine
DMBA	Dimethyl-benz[a]anthracene
DNA	Deoxyribonucleic acid

DOTMA	N-[1-2,3-dioleyloxy)propyl]-N,N,N-trimethlyammonium chloride
dpm	Disintegrations per minute
ECVAM	European Center for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EGA	Ethylene glycol adipate
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
EROD	Ethoxyresorufin-o-deethylase
ETC	Electron transport chain
FABP	Fatty acid binding protein
FAD	Flavin adenine dinucleotide
FADH$_2$	Flavin adenine dinucleotide, reduced form
FCS	Fetal calf serum
FID	Flame ionisation detector
GCMS	Gas chromatography-mass spectrometry
GLC	Gas liquid chromatograph
GPx	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
H$_2$O$_2$	Hydrogen peroxide

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

mV	Millivolts
mw	Molecular weight
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide dinucleotide phosphate, oxidised form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
NAR	Nagase analbuminemic rats
NMR	Nuclear magnetic resonance
O_2	Superoxide anion
ODC	Ornithine decarboxylase
PBS	Phosphate buffered saline
PLPC	1-Palmitoyl-2-linoleoyl phosphatidylcholine
polyHEMA	Polyhydroxymethylmethacrylate
PUFA	Polyunsaturated fatty acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid

TCA cycle	Tricarboxylic acid cycle
U	Units
UDPGT	Uridine diphosphate glucuronyltransferase
UV	Ultraviolet
V v v	Volume per volume per volume
VLDL	Very low density lipoprotein
V_s	Sample volume
V_t	Total volume
W/v	Weight per volume
WHHL	Watanabe Heritable Hyperlipidemic

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CHAPTER 1

Introduction

1 1 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 1 1 1, where it is attached to the diaphragm and

protected by the ribs, see Fig 1 1 2 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 1 1 3 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 1 1 1 Site of the human liver
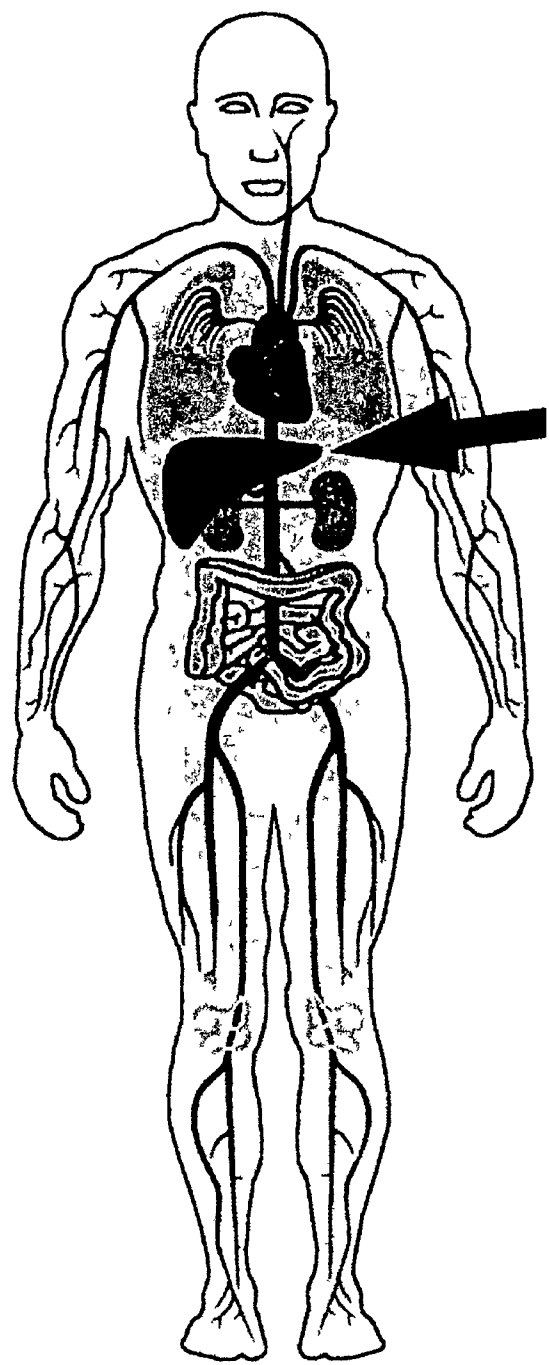
The arrow indicates the liver

Fig.1.1.2. Human liver, *in situ*

Right lung   Heart   Left lung

Diaphragm

Liver

Gall Bladder

Large intestine

Small intestine

Stomach

Spleen
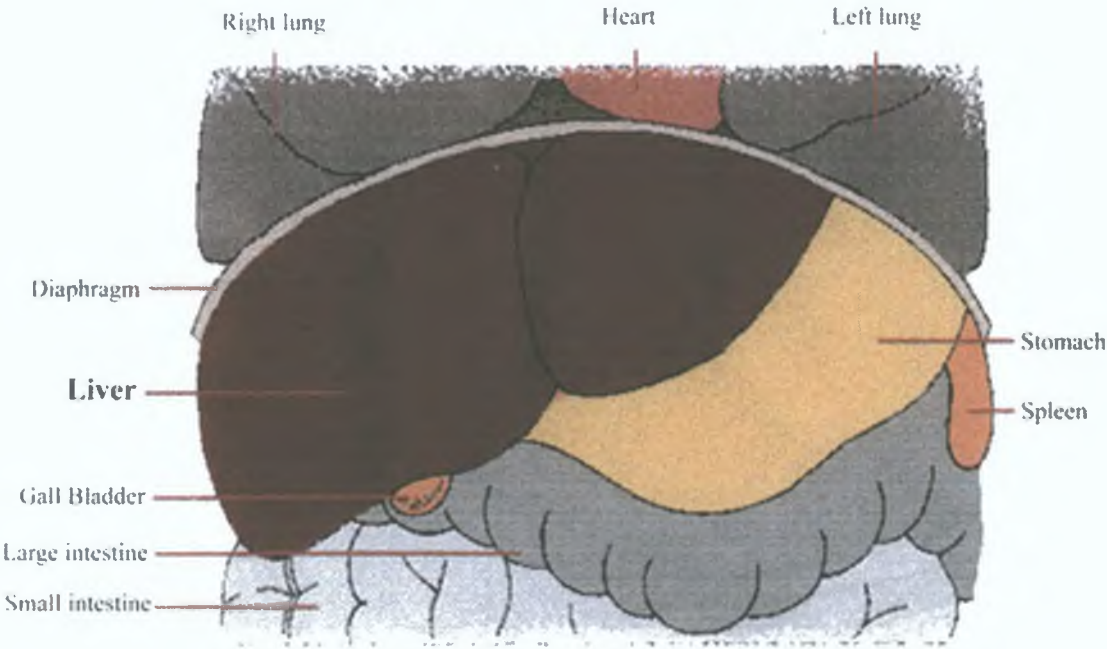
Reproduced from Microsoft Encarta 98 Encyclopedia.

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.
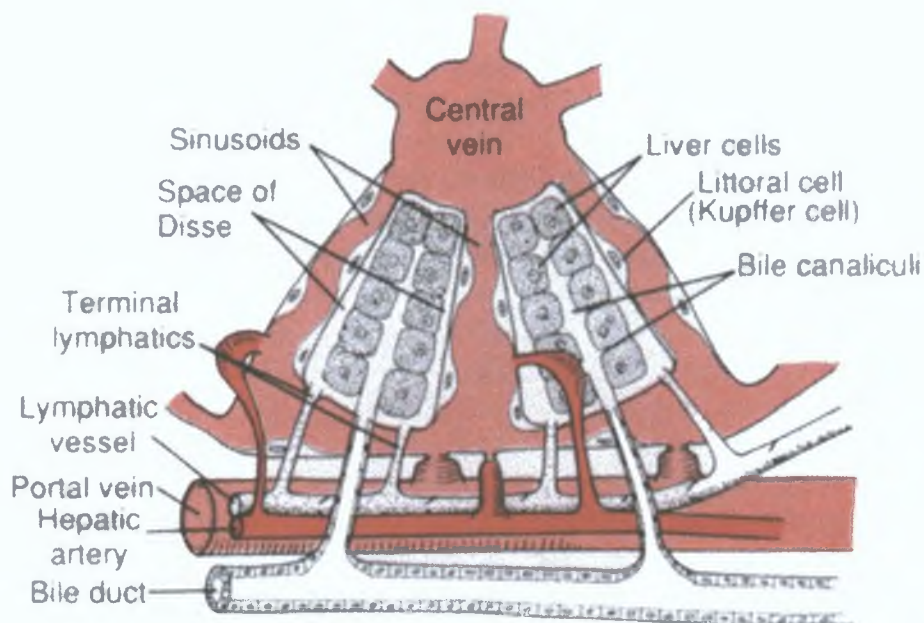
Reproduced from Guyton and Hall (1996), Textbook of Medical Physiology (9th Edition).

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 μm, 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 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

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 1 1 4) Stored glycogen can maintain the blood glucose level for 24

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Fig 1.1.4 The Glucose buffer function of the liver

GLUCOSE RETENTION   GLUCOSE RELEASE

insulin

high blood sugar
     (food)   → HEPATOCYTE

lactic acid   O₂

glycogen
breakdown

gluco
neogenesis

{ low blood sugar
  adrenaline
  noradrenaline
  glucagon

{ hydrocortisone
  cortisone
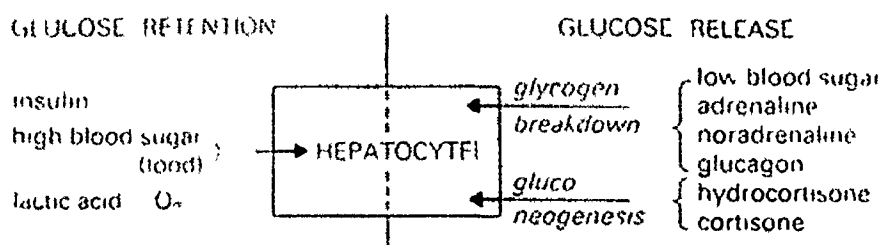
Reproduced from Horner Andrews (1979), Liver

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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 Co A is formed which becomes part of the tricarboxylic acid (TCA) cycle The liver cannot use all the acetyl Co A 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 Co A 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, B_{12}, D, E and K are stored in the liver The supply of vitamin A would last for 10 months where as D would last 3 to 4 months and that of B_{12} 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

Fig. 1 1.5   The role of the liver in lipid synthesis and metabolism.
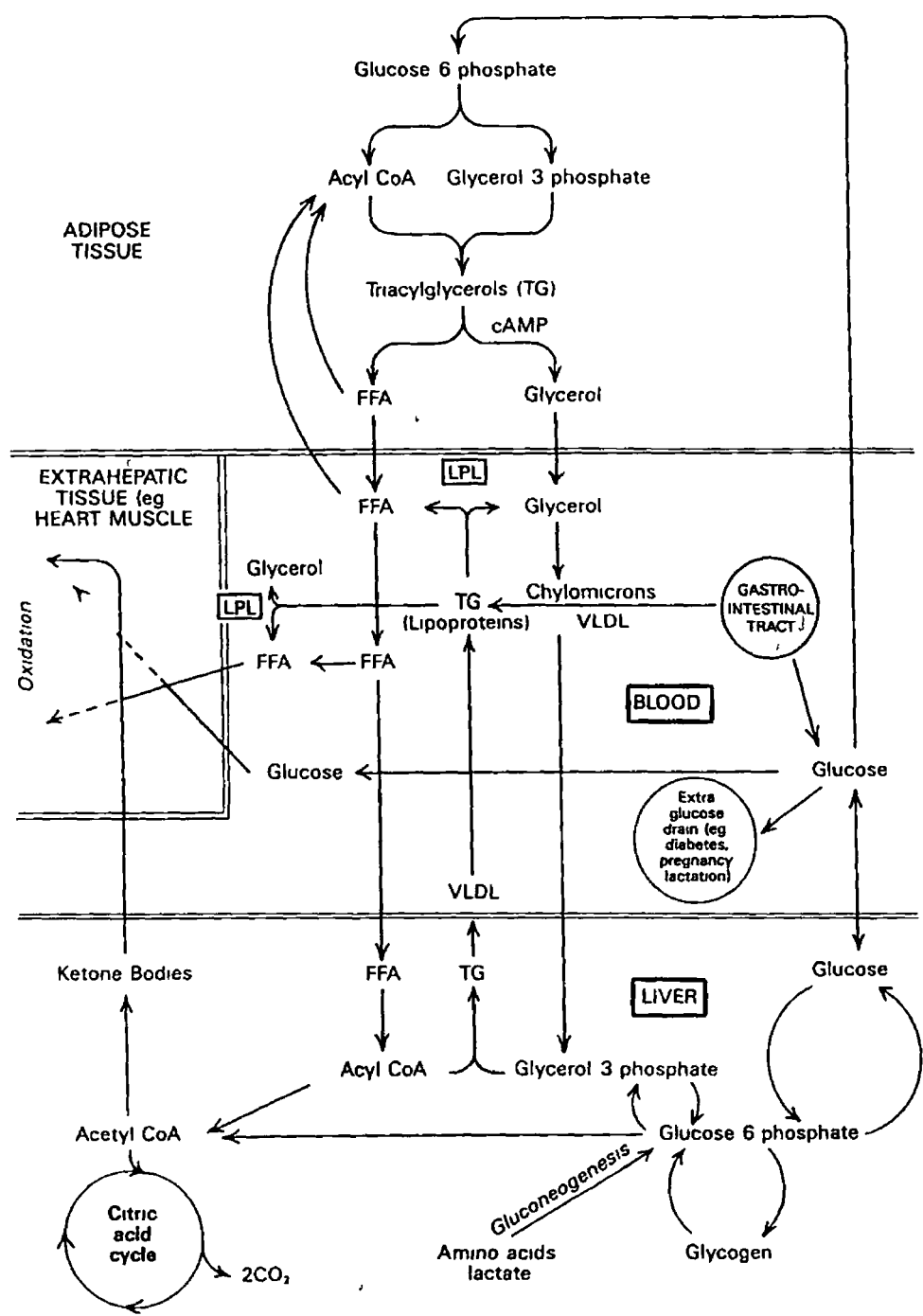
Reproduced from Harper's Biochemistry (1996)

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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

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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 ^{125}I-labeled or ^{3}H-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 Shcer and have been used to

compare the metabolism of cyclosporine A in different species (Vickers, 1992) Species differences in coumarin 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, cyproterane 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

literature 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% CO_2 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 ^{14}C HMG CoA to ^{14}C mevalonate Both compounds were found to induce a dose-dependent decrease in cholesterol biosynthesis (Gebhardt et al, 1994) α-Amanitin and dl-propangylglycine 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 adeno-viral 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-dioleoyloxy)propyl]-N,N,N-trimethlyammonium 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)

Raper *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 DiI-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

- 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 1 4 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 1 4 2 Wang (1995) advocates "resuscitating" hepatocytes prior to transplantation This involves culturing the hepatocytes for three days with a hepatotrophic factor such as

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Fig 1 4 1 Hepatocytes immobilised on microcarriers
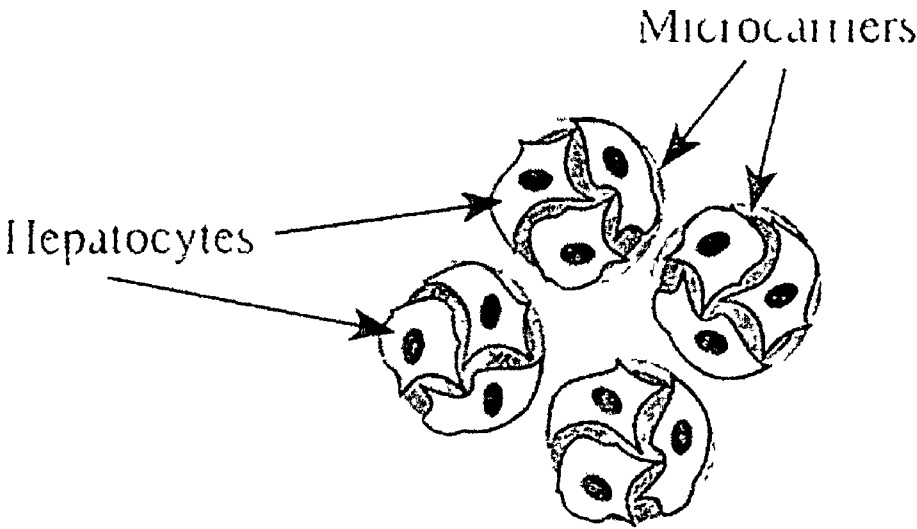
Fig. 1 4 2 Microencapsulated hepatocytes

IMPERMEABLE SUBSTANCES
e g Hepatocytes, Lymphocytes, Antibodies Bacteria etc

PERMEABLE SUBSTRATES (S), PRODUCTS (P)
e g Ammonia, Phenols Mercaptans Fatty Acids Bile Salts
    Albumin, Clotting Factors etc

Reproduced from Dixit (1995)

28
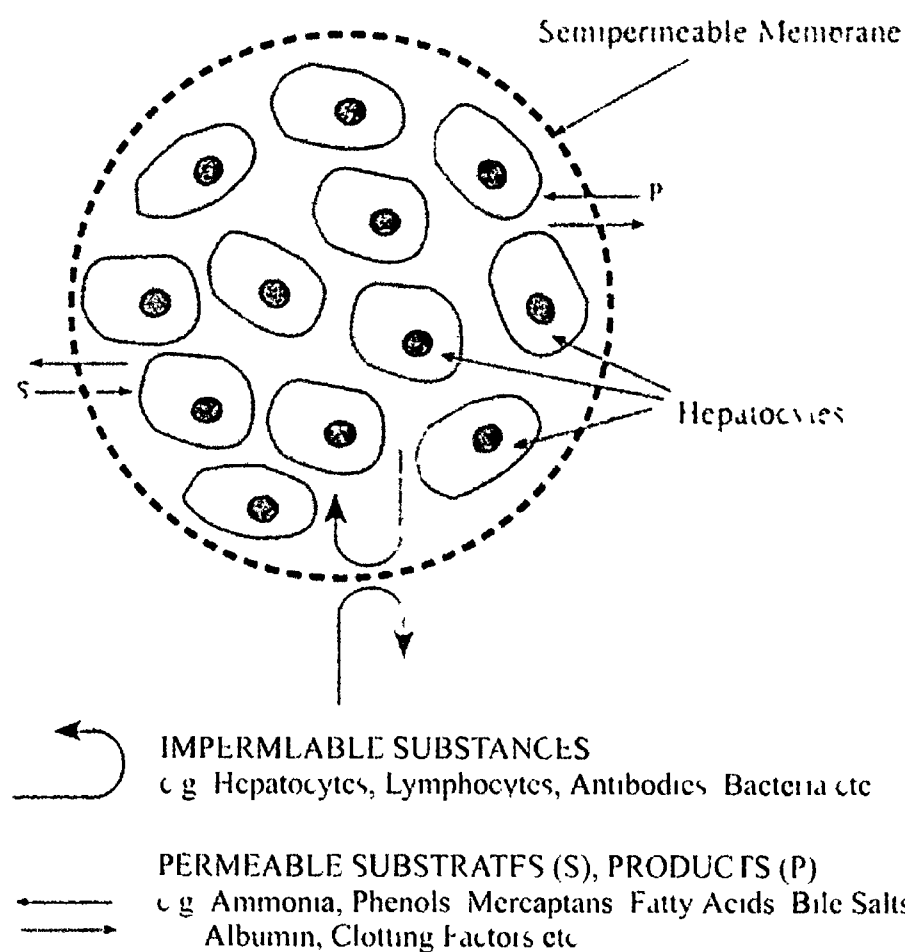
insulin Hepatocytes would therefore have time to recover from the trauma suffered during isolation and become fully functional again

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 1 4 3

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 1 4 3 Capillary hollow fiber hepatocyte bioreactor
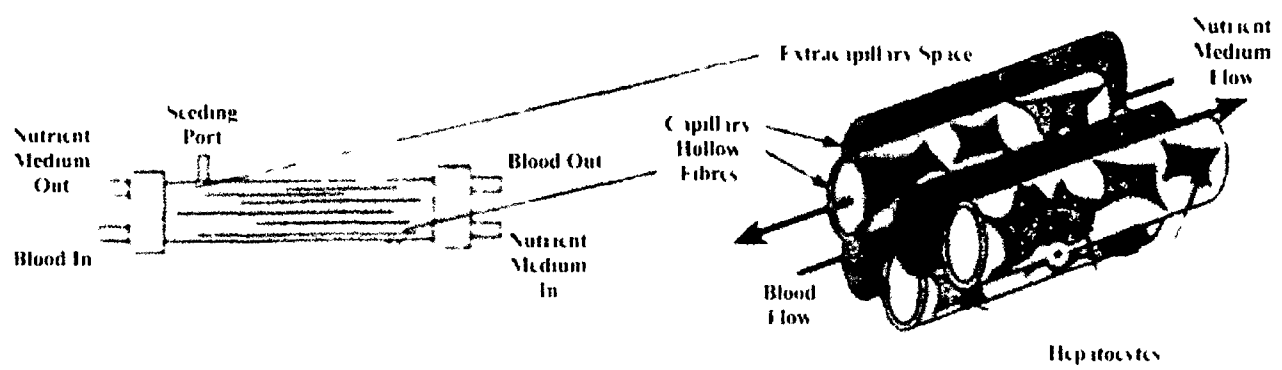
Reproduced from Dixit (1995)

microcarriers 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 repurfusion 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), sulfhydryl compounds and quinones or quinone-like substances The most important source of radical species in biological systems, however, is O_2 (Singal *et al*, 1988) At

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53 8% 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 1 5 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

$$A_x{}^xB \longrightarrow A^x + B_x$$
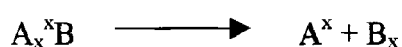
Heterolytic fission, where both electrons remain with one atom, results in ion *formation*

$$A_x{}^xB \longrightarrow A_x{}^x + B^+$$
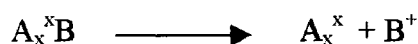
Oxygen readily accepts electrons from other substances – it is a good oxidising agent, but

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 π^* and σ^* orbitals

where as in singlet oxygen both electrons are in the π^* 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

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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

```
R
 \                           R H
  CH                         |/
   \\                        /\
    CH                      O  CH
    |    + O₂ (singlet) ──►  |   ||
    CH                      O  CH
   //                        \ /
  CH                         / \
  |                         R  H
  R
```
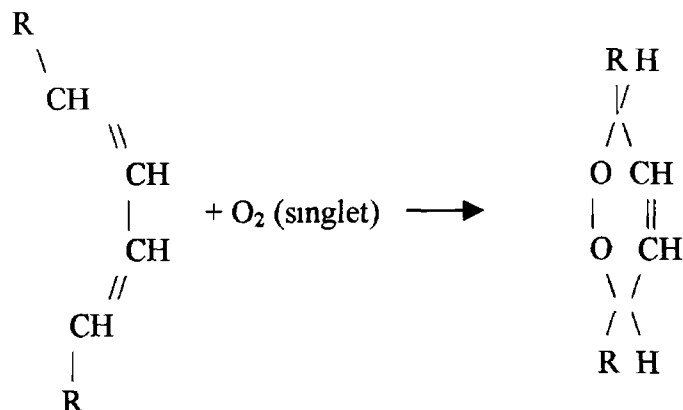
Reaction with conjugated double bond to form an endoperoxide

```
CH₂                         CH₂
 //                        /    \
 CH       + O₂ (singlet) ──► CH   O
 |                          ||    |
 CH₂                        CH₂   O
  \                              /
   H                            H
```
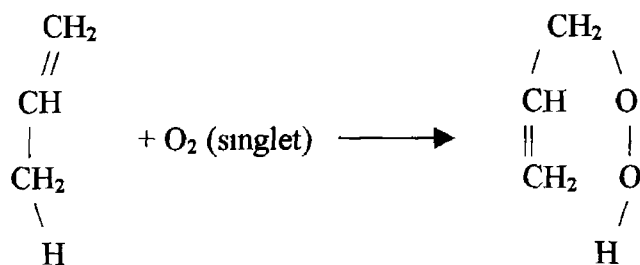
Reaction with a double bond to form a hydroperoxide

```
                                                              \/
 |                    |                                        N
 CH                   HC----O         |                        |
 ||  + O₂ (singlet) ──► |    |  ──►  HC----O  +   H---C
 CH                   HC----O                                  ||
 /                    /                                        O
 N                    N
 /                    /
```
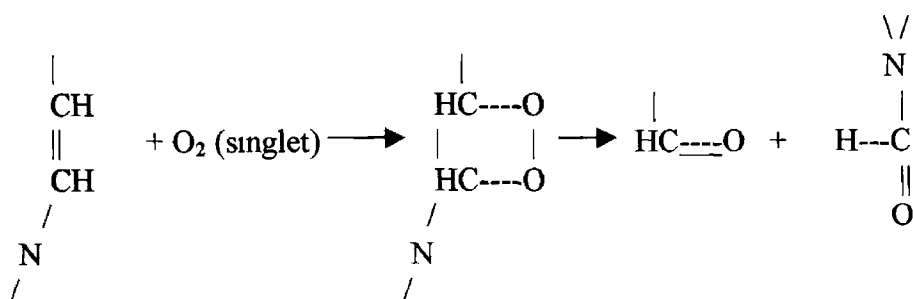
Reaction with a double bond adjacent to an electron-donating atom to form carbonyl compounds via a dioxetane intermediate

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Fig. 1.5.1 Reactive oxygen species and cell Injury
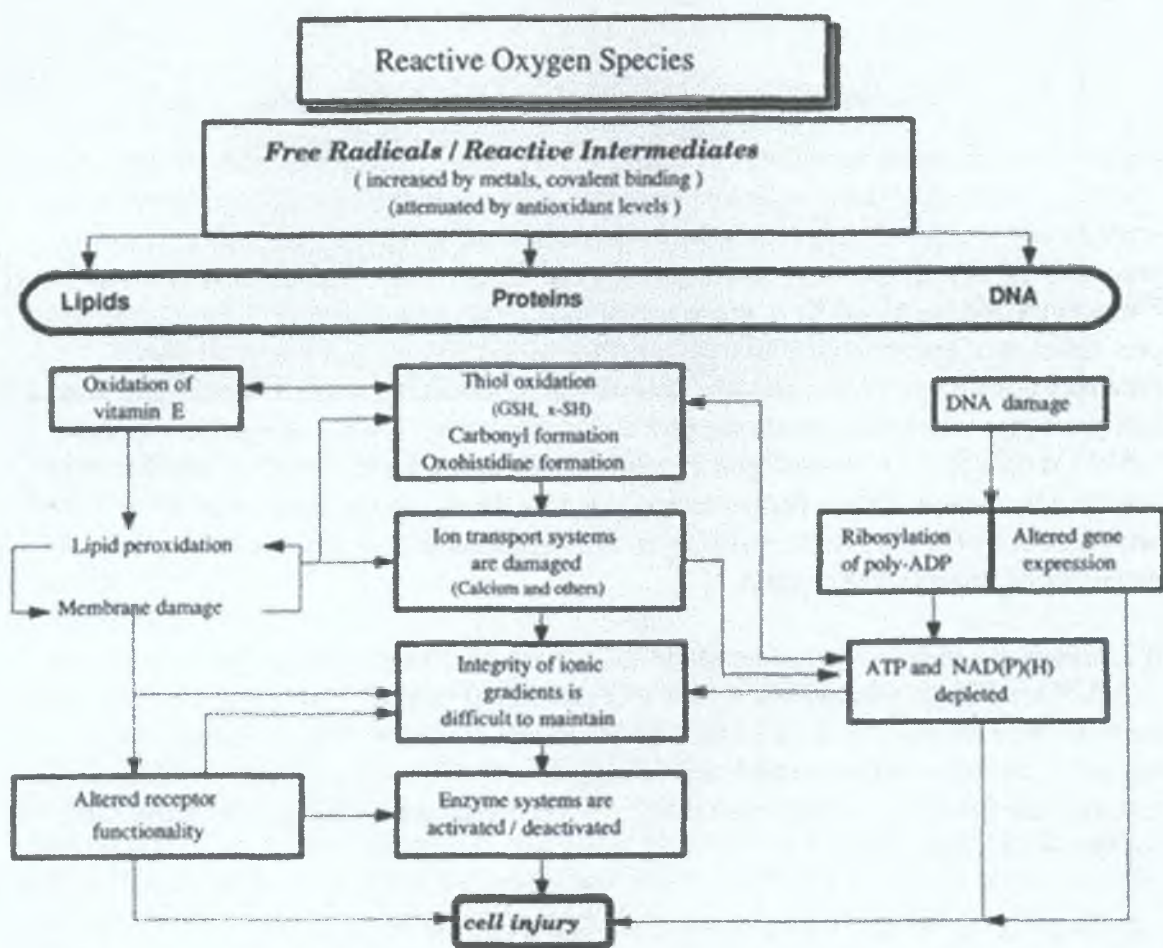
Reproduced from Acworth and Bailey (1995).

Formation and reactions of the superoxide radical

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

$$O_2 + e^- \longrightarrow O_2^{\cdot}$$
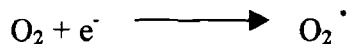
It has one unpaired electron and is therefore less of a radical than oxygen It shall henceforth be denoted by O_2^- It is stable in both aqueous and organic environments although it's chemical properties differ in each In aqueous solution it can accept a proton and form the hydroperoxyl radical $HO_2^{\cdot}$ but at the pH of body fluids most remains as 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 α tocopherol to the tocopheryl radical as shown below
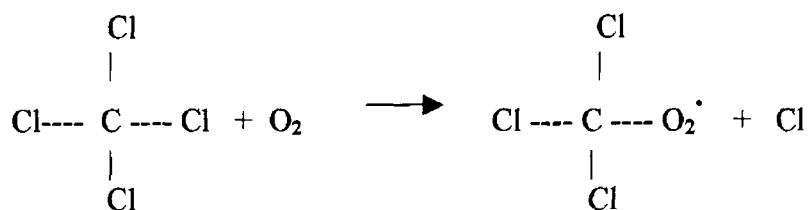
Reaction with carbon tetrachloride to produce a peroxyl radical

$$O_2 \ + \ \text{tocopherol} \ \longrightarrow \ \text{tocopherol ion} \ + \ HO_2^{\bullet}$$

$$HO_2^{\bullet} \ + \ \text{tocopherol} \ \longrightarrow \ H_2O_2 \ + \ \text{tocopheryl radical}^{\bullet}$$

$$O_2 \ + \ \text{tocopherol ion}^{-} \ \longrightarrow \ O_2^{-} \ + \ \text{tocopheryl radical}^{\bullet}$$

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 $FADH_2$ 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 1 5 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

NADH $\longrightarrow$ $2e^-$ NADH-
NAD$^+$ $\longleftarrow$ coenzyme Q reductase

E — FADH$_2$
(protein-bound)

C
O
E
N
Z
Y
M
E

Q

P
O
O
L

$$H_3CO \quad O \quad CH_3$$
$$H_3CO \quad (CH_2CH=C-CH_2)_{10}H$$
$$O$$
with CH_3 on the isoprenoid unit

Oxidized Q

$\downarrow$ $1e^-,1H^+$

Partially reduced (semiquinone)

$\downarrow$ $1e^-,H^+$

Fully reduced (Co QH$_2$)

CoQH$_2$ — cytochrome c reductase

Cytochrome c

Cytochrome oxidase

O$_2$ $\searrow$ 2H$_2$O (per 4e$^-$ taken in)
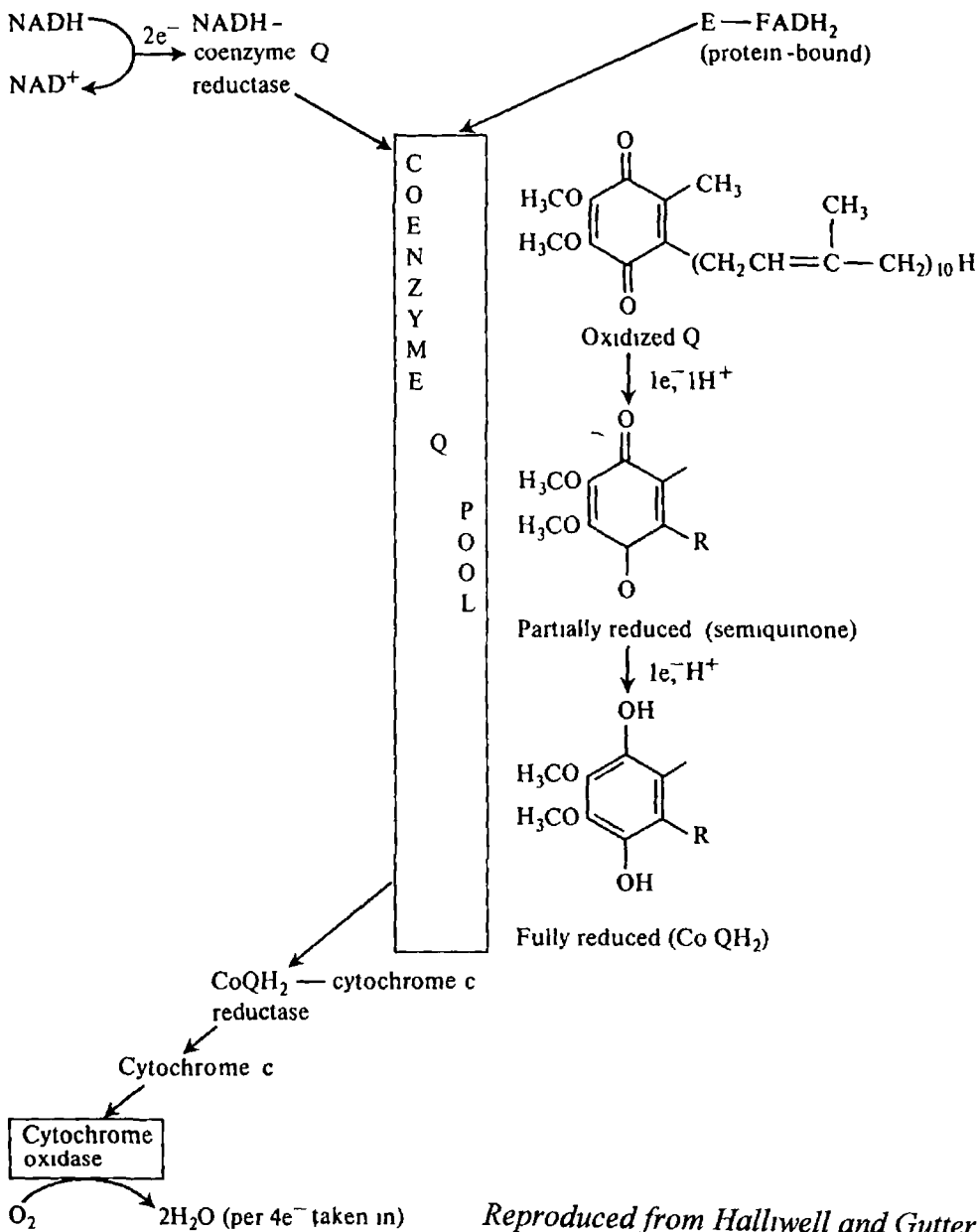
Reproduced from Halliwell and Gutteridge (1989)

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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

$$AH + O_2 + RH_2 \longrightarrow A\overset{.}{O}H + R + H_2O$$
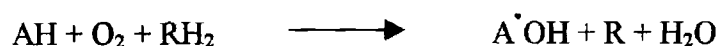
where AH is the substrate and RH_2 is the reducing agent Further metabolism may then occur (as described in section 1 1) In the liver the electrons required for cytochrome P450 function are donated by NADPH via NADPH-cytochrome P450 reductase This enzyme can leak electrons to oxygen, reducing it to form 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

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in turn passes the electrons to desaturase Electrons may be leaked to oxygen from both cytochrome b_5 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 O_2^{2-} 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, $OH^\bullet$ and is shown below

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^\bullet$$
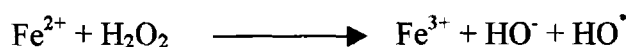
$OH^\bullet$ is one of the most reactive species known and reacts with almost every type of molecule found in living cells Due to its high reactivity, any hydroxyl radical formed will react at or very close to its site of formation, and although the radicals it forms are usually less reactive, most of the damage is done through further reactions of these

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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 1 5 3

Other radicals found in biological systems include thiyl, carbon centred, nitrogen centred and phosphorous centred radicals

Fig 1 5 3 The Formation of Reactive Oxygen Species
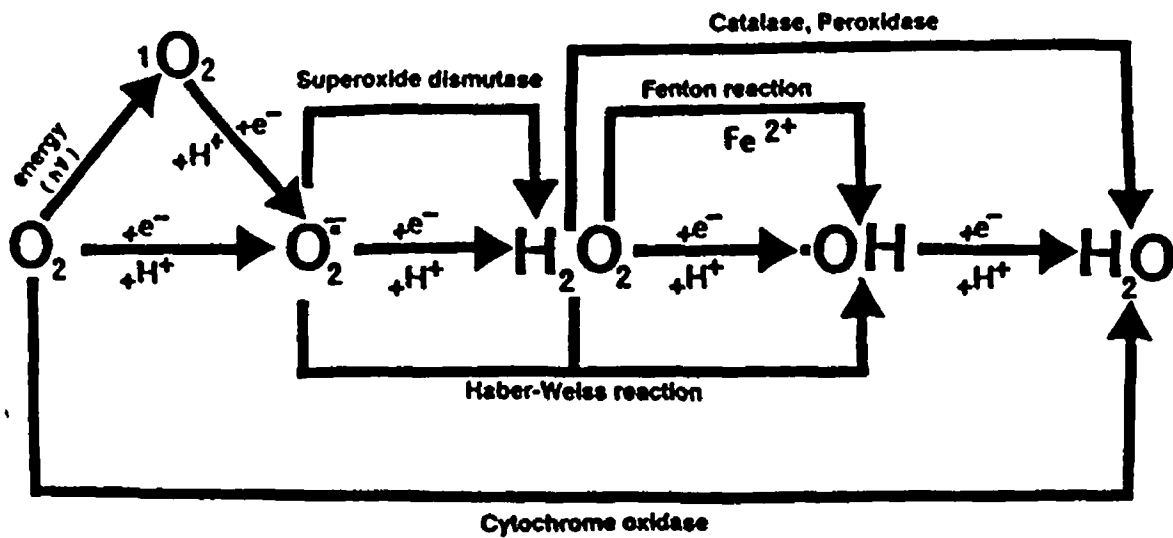
Biological functions of free radicals

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

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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 1 5 4

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 1 7 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)

1 6 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

Glycolysis/Krebs Cycle   Pentose Cycle/Lipid oxidation

O_2 *Quinone*   NADH   NADPH   2GSH

NAD(P)H Reductase   GSSG Reductase

O_2^- *Semiquinone* NAD^+ $NADP^+$ GSSG

NAD(P)H / NAD(P)$^+$

SOD

Ca^{++} release

Lipid peroxidation

Catalase
$O_2 \leftarrow H_2O_2$ $M^+ \rightarrow OH^- + OH$ ROOH   GSSG

GSH

GSH Peroxidase

GSH Peroxidase

GSSG

ROH   GSH

$2H_2O$
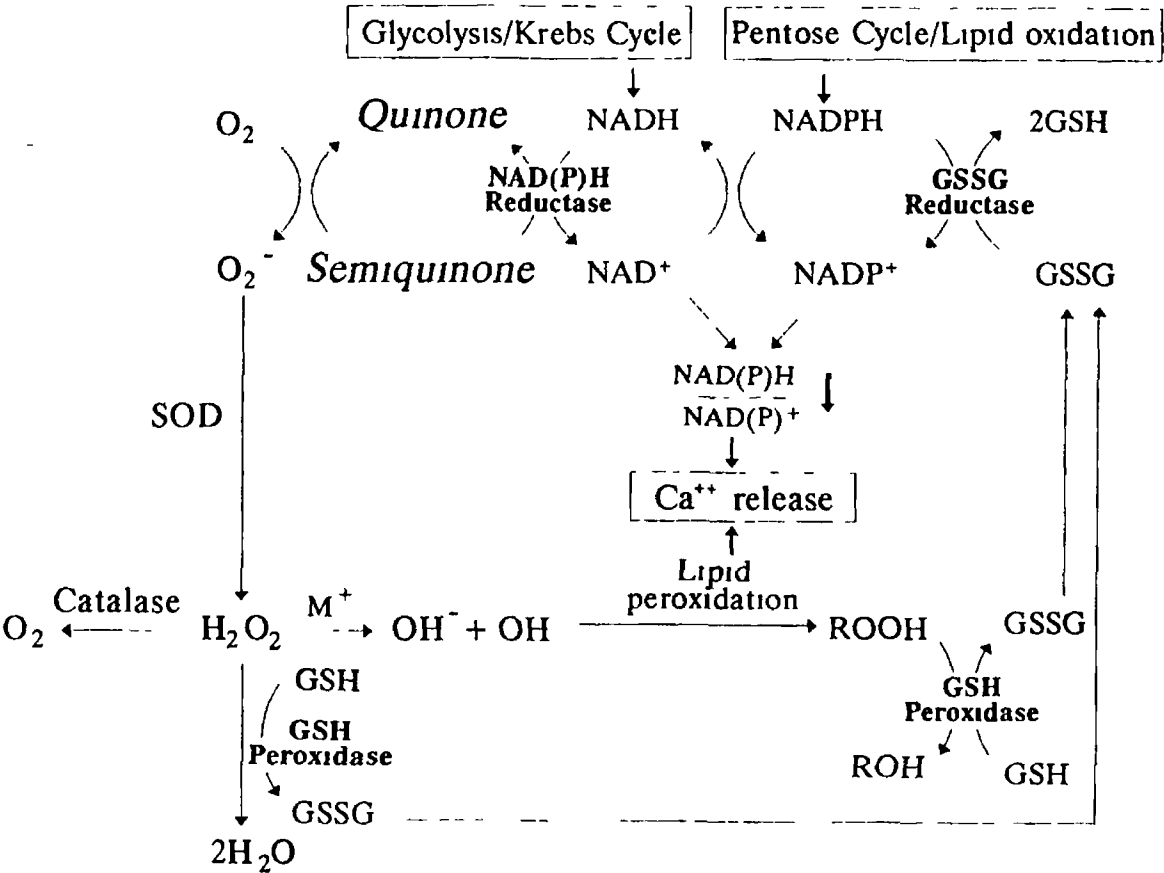
Reproduced from Castell et al (1997)

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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 1 6 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 1 Lipid Peroxidation

Fatty acid with
3 double bonds

Hydrogen abstraction

$-H^\bullet$

Molecular rearrangement

Conjugated diene with UV
absorbance at 234 nm

O_2 Oxygen uptake

Peroxy radical abstracts
$H^\bullet$ from another fatty acid causing
an autocatalytic chain reaction

$H^\bullet$

Lipid
hydroperoxide

↓

Cyclic
peroxide

↓

Cyclic
endoperoxide

Fragmentation to aldehydes
(including malondialdehyde)
& polymerization products
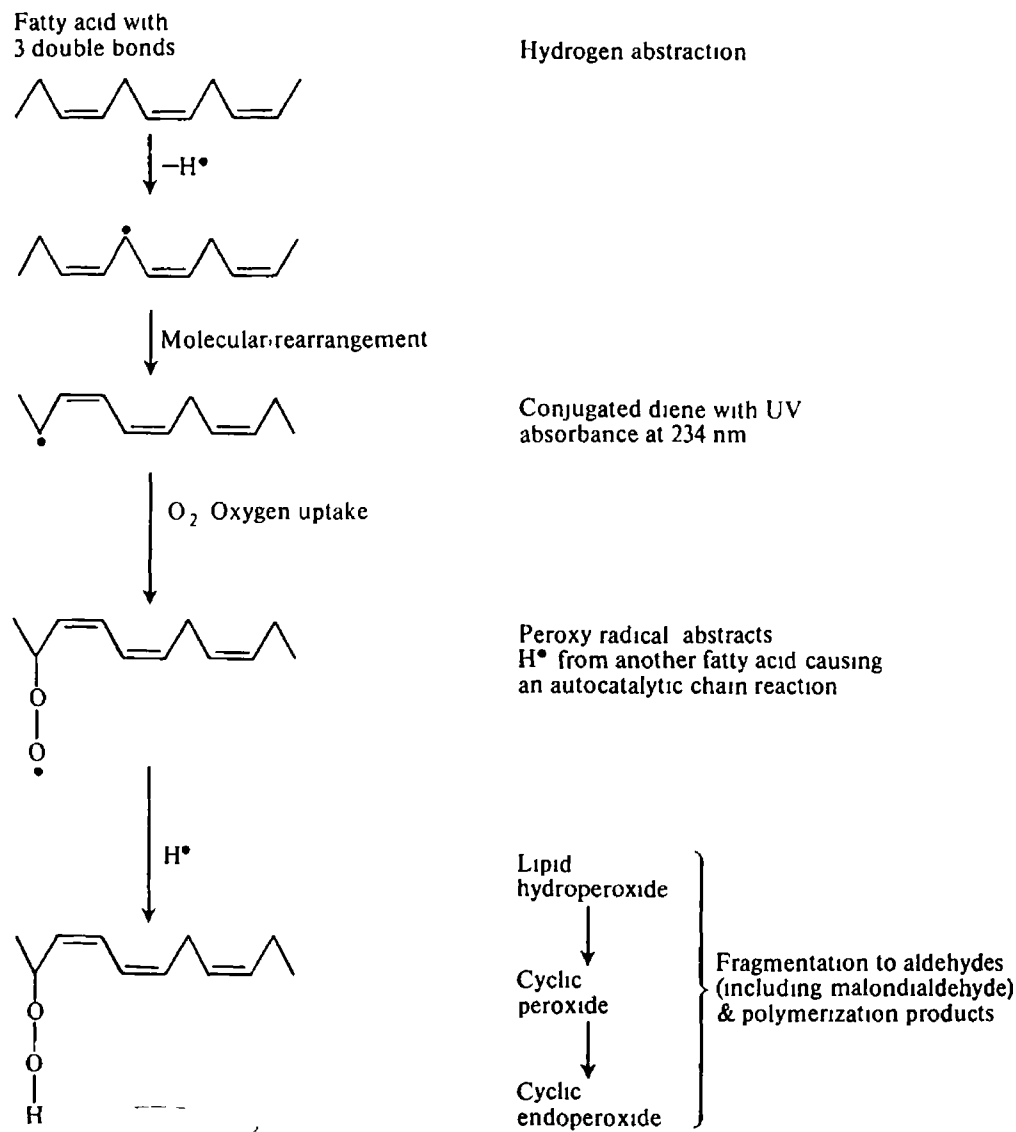
Reproduced from Halliwell and Gutteridge (1989)

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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 1 7 1

Antioxidant Enzymes

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

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$
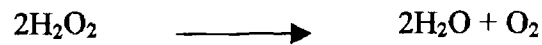
The oxygen produced in this reaction is in the ground state, effectively stopping any further radical activity In animals, it is found in all organs, especially the liver and erythrocytes It is composed of four protein subunits Each subunit contains a haem group bound to the active site and usually one bound NADPH molecule The mechanism of reaction is thought to be as follows

$$Catalase - Fe(III) + H_2O_2 \longrightarrow compound\ I$$
$$Compound\ I + H_2O_2 \longrightarrow catalase - Fe(III) + H_2O + O_2$$
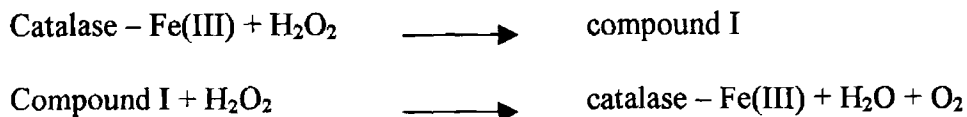
The structure of compound I is not known as the extensive delocalisation of the charge over the haem rings makes it very difficult to determine Catalase has a very high affinity for the destruction of hydrogen peroxide and in the presence of a steady supply of

Fig 1 7 1 Cellular antioxidant defense

Vitamin E
β carotene
ENDOPLASMIC RETICULUM
NUCLEUS
DNA
Vitamins C and E
β-carotene
LYSOSOMES
PEROXISOMES
CYTOPLASM
Catalase
GSH
MITOCHONDRION
Glutathione
Peroxidase
Cu/Zn
SOD
Vitamin C
LIPID BILAYER OF ALL
CELLULAR MEMBRANES
Vitamin E
Mn
Vitamin E +
β carotene
SOD + Glutathione Peroxidase
+ GSH
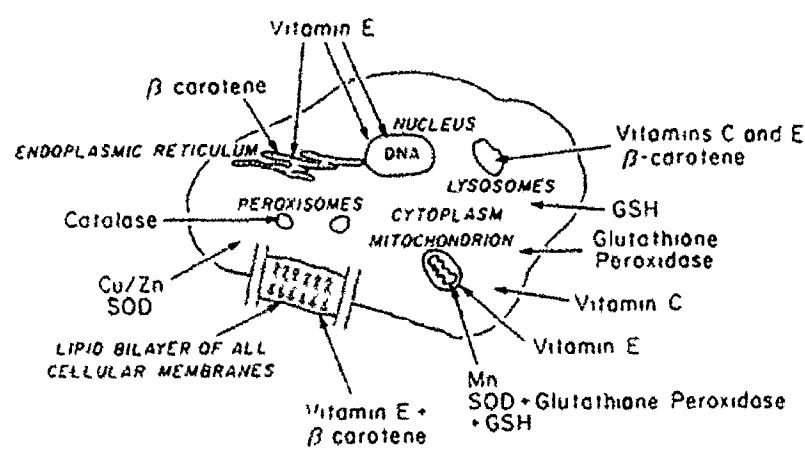
hydrogen peroxide, can also have some peroxidase - like activity due to the action of compound I Compound I can oxidise ethanol, methanol, acetaldehyde, nitrous oxide and possibly elemental mercury If the catalase subunits are separated, they have a wider range of peroxidase activity Catalase activity 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

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O$$

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 1 7 2

Fig 1 7 2 The structure of reduced and oxidised glutathione

$$
\begin{array}{c}
O \quad O \\
\backslash\backslash \quad / \\
C \\
| \\
H-C-H \\
| \\
N-H \\
\backslash \\
O{=}C \\
| \\
H-C-CH_2-SH \\
| \\
N-H \\
| \\
O{=}C \\
| \\
H-C-H \\
| \\
H-C-H \\
| \\
{}^{+}H_3N-C-H \\
| \\
C \\
// \quad \backslash \\
O \quad O
\end{array}
\qquad
\begin{array}{l}
Gly \\
\\
Cys \\
\\
Glu
\end{array}
$$

GSH

$$
\begin{array}{ccc}
Gly & & Gly \\
| & & | \\
| & & | \\
| & & | \\
Cys & - S - S - & Cys \\
| & & | \\
| & & | \\
| & & | \\
Glu & & Glu
\end{array}
$$

GSSG

53
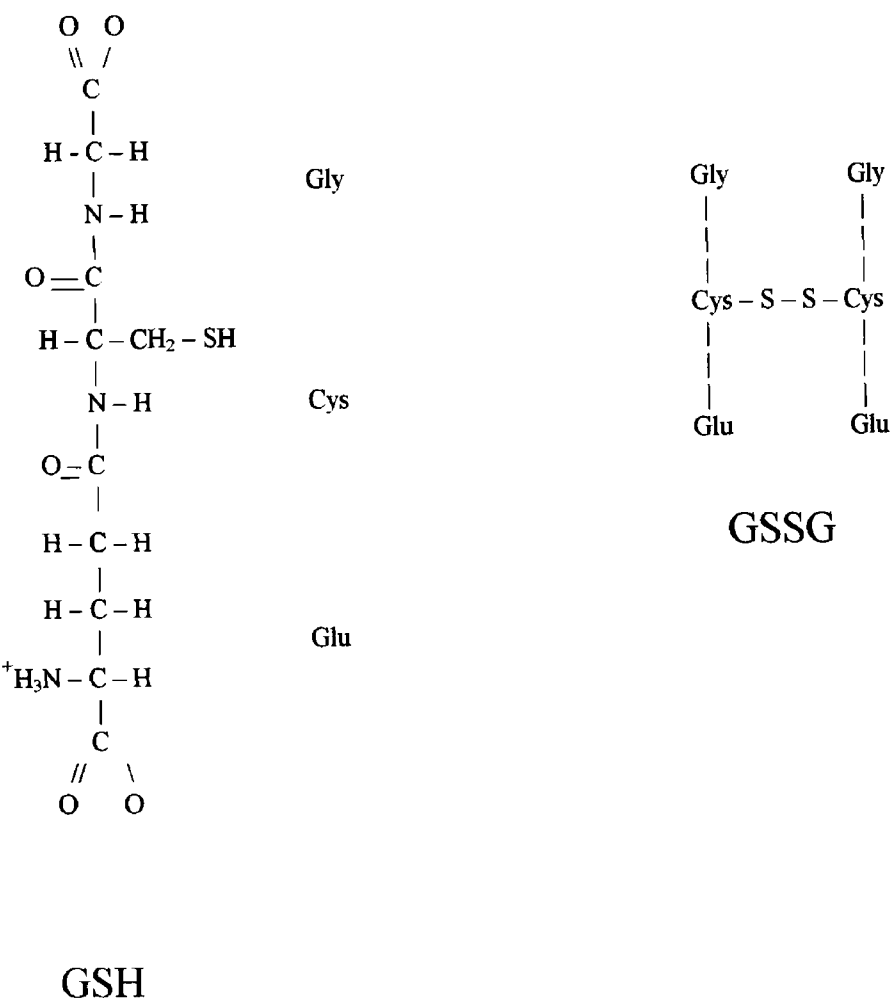
Glutathione reductase reduces the oxidised glutathione produced by GPx ensuring a steady concentration of reduced glutathione in the cell The reaction is as follows

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

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

$$Enz - Cu^{2+} + O_2 \longrightarrow Enz - Cu^+ + O_2$$

$$Enz - Cu^+ + O_2 + 2H^+ \longrightarrow Enz - Cu^{2+} + H_2O_2$$
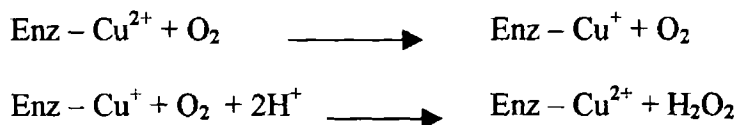
MnSOD is a less stable enzyme than CuZnSOD It is also heat labile and the activity decreases at alkaline pH It is, however, insensitive to cyanide MnSOD comprises 10% of total SOD in rat liver, while the percentage in human liver is higher (Halliwell and Gutteridge, 1989) It is situated in the mitochondrial 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

$$2O_2 + 2H^+ \longrightarrow H_2O_2 + O_2$$

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

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 oxidised, 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 thiyl 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)

Fig 1 7.3 The structure of ascorbic acid and dehydroascorbate

Ascorbate   Dehydroascorbate
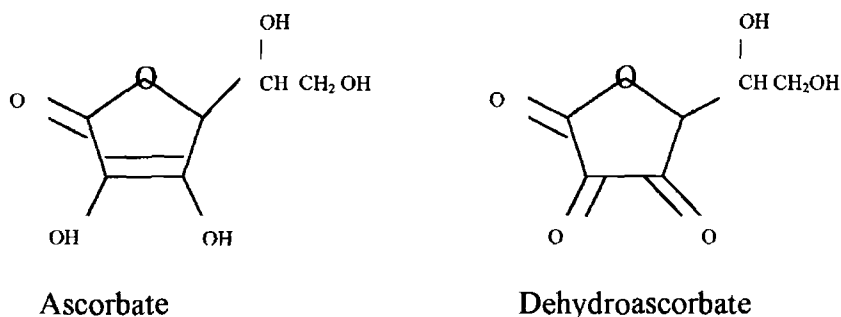
Ghosh *et al* (1997) studied lipid peroxidation in guinea pig microsomes due to NADPH-initiated cytochrome P450 activity, in the absence of free iron After a 30 minute period the conjugated dienes and lipid hydroperoxide produced were measured A time-dependent increase in both these parameters was observed 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-Fe^{3+} 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), mannitol (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

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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 $Fe^{3+} O_2^-$

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 $Fe^{3+} O_2$

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

$$R\text{---}\overset{\textstyle\backslash}{\underset{\textstyle/}{C}}\text{---}H \ + \ OH \ \longrightarrow \ R\text{---}\overset{\textstyle\backslash}{\underset{\textstyle/}{C}} \ + \ H_2O$$

$$R\text{---}\overset{\textstyle\backslash}{\underset{\textstyle/}{C}} \ + \ O_2 \ \longrightarrow \ R\text{---}\overset{\textstyle\backslash}{\underset{\textstyle/}{C}}O_2$$

Urate Peroxyl radical

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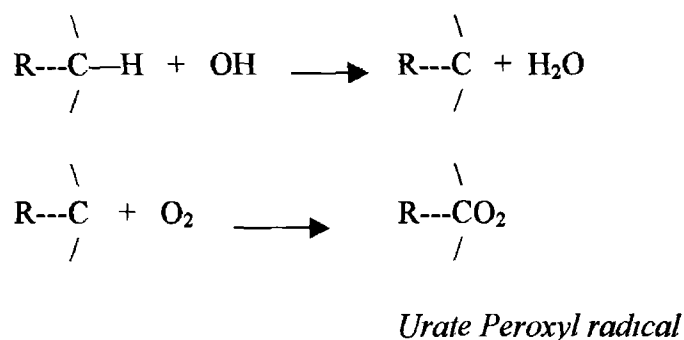
Metallothioneins   Metallothioneins are proteins with a molecular weight of approximately 6500 They are found in the cytosol of eukaryotic cells especially the liver, kidney and gut They are sulphur rich and can bind and store metal ions Binding is achieved by association of the cysteine –SH groups with 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 1 7 5

Fig 1 7.5 The structure of β carotene
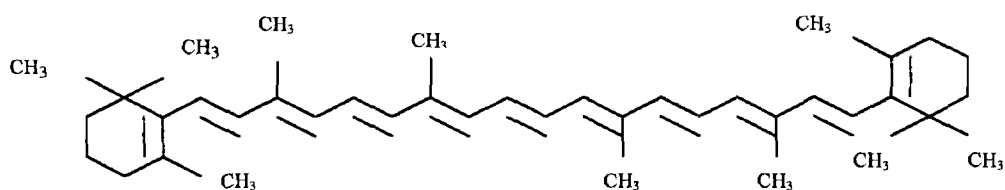
The Physician's Health Study which was commenced in 1982 involved the study of the health benefits of dietary β carotene Administration of 50 mg β carotene on alternate days resulted in a 44% reduction in major coronary events i e myocardial infarction, revascularisation and death (Rice-Evans, 1995) 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)

α Tocopherol   α Tocopherol, also known as vitamin E although vitamin E is really the

collective name for a group of tocopherols - α, β, γ, δ of which α 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) α 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 *et al*, 1994) as shown

$$ROO + AH \longrightarrow ROOH + A \qquad \text{reaction 1}$$
$$ROO + A \longrightarrow ROOA \qquad \text{reaction 2}$$

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

Fig 1.7 6 The structure of α tocopherol
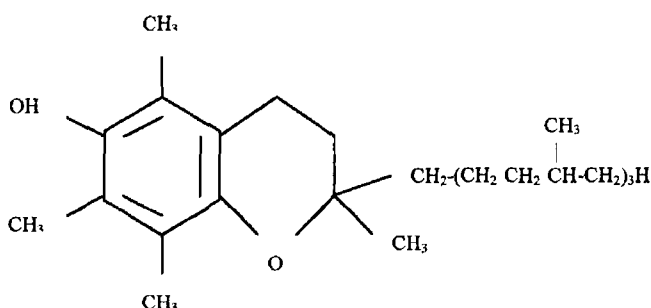
With relation to α tocopherol, the second ring holds the oxygen so that the p-type lone pair makes an angle of approximately 74° with the aromatic ring The orbital of the lone pair can overlap the orbital containing the unpaired electron and so stabilize the phenoxyl radical The stability of this radical 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 nitrites 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 erythrocyte 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 1 7 7

Fig 1 7 7 Antioxidant interaction
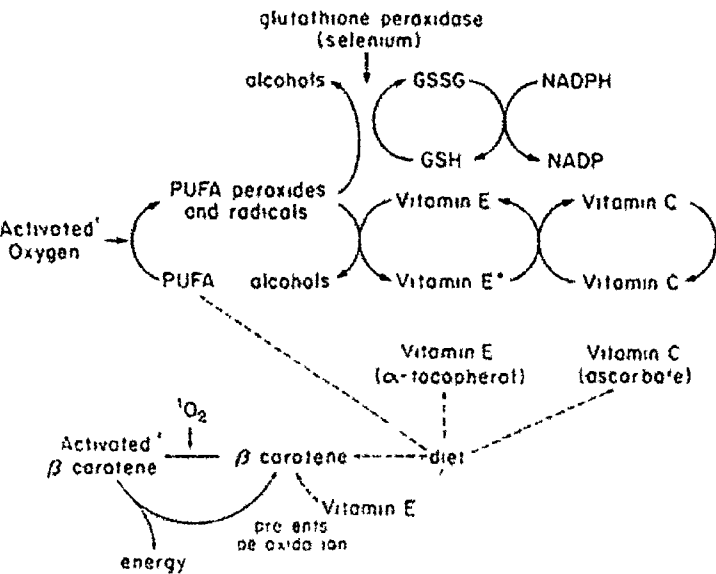
Reproduced from Machlin and Bendich (1987)

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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,

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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 _cis_9, _trans_11 isomer of CLA
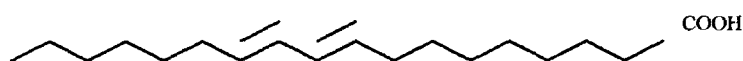
The fact that Ha's research (1990) into the antioxidant properties of CLA had been carried out in a phosphate buffer ethanol mixture, which does not reflect physiological conditions and that Ip's work (1991) yielded evidence that CLA's antioxidant activity was specific to mammary tissue and that a lower 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'-azo*bis*(2,4-dimethylvaleronitrile) and 2,2'-azo*bis*(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 deterioration, 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*, 1991)

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 CO_2 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% CO_2 and 95% O_2 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, cholestanetriol, 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 [^{3}H] mevalonolactone (33 Ci/mmol), 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA (45 6 mCi/mmol), L-[4,5-^{3}H]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

Labels: clamp, outflow cannula, rib cage, right atrium, heart, interior vena cava, ligature, liver, ligature, kidney, wall, severed interior vena cava, retractor, inflow cannula (inside portal vein), clamp
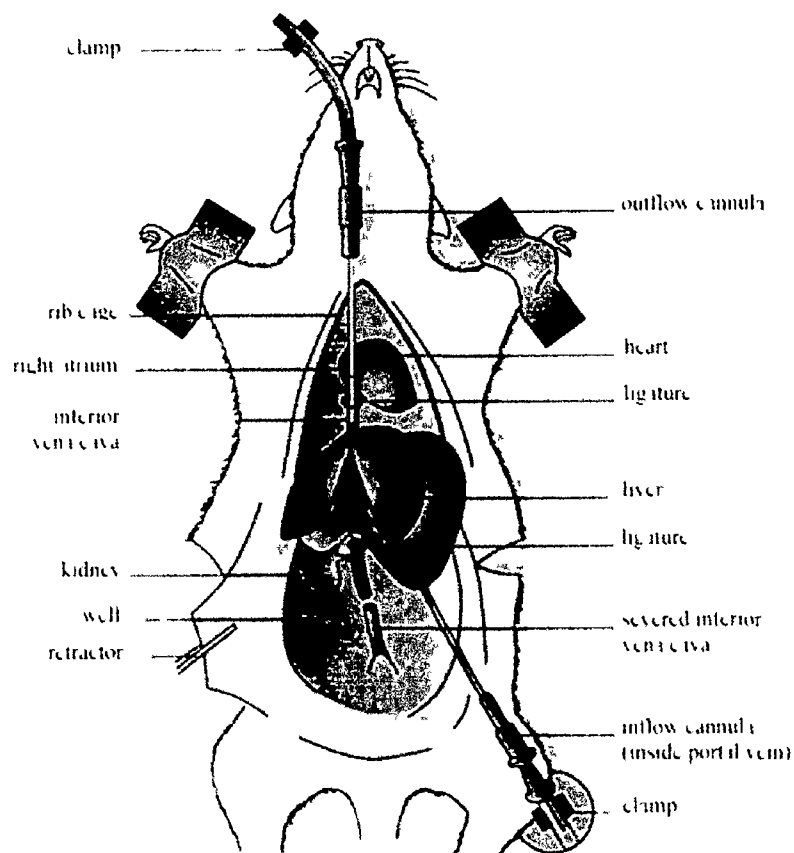
Fig 2.3.1b Liver perfusion apparatus
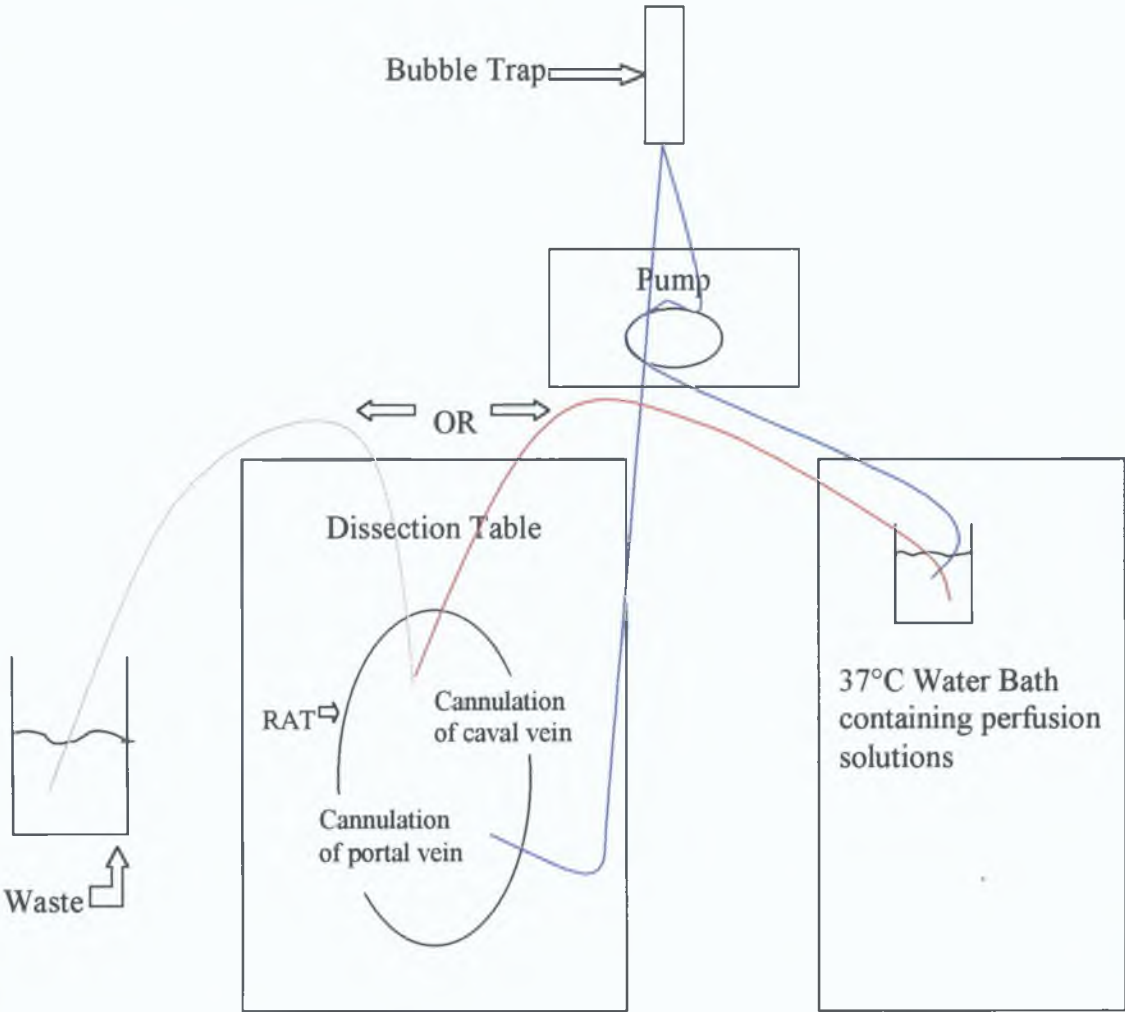
Liver perfusion apparatus, showing temperature control (water bath maintaining perfusion solutions at 37℃), bubble trap, dissection table, pump, waste container and tubing. Blue tubing is that going to the liver. Red tubing is that going from the liver to waste – in order to flush out blood and finally, 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 1×10^{-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

Fig 2 3.1c Procedure for *In Situ* Recirculating Perfusion

Sprague Dawley rat (250 g)

sacrifice via halothane anesthesia and cervical dislocation

↓

Cannulation

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

↓

Two step perfusion

1   Hanks' BSS containing EGTA

2   Hanks' BSS containing calcium and collagenase

pH 7 4, temperature 37°C

↓

Removal of Debris

Filter through nylon mesh with protein-containing Hanks' BSS

↓

Wash X 3 in PBS

Percoll treatment

↓

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

$$\text{Pyruvate} + \beta\text{NADH} \quad \rightarrow \quad \text{lactate} + \beta\text{NAD}$$

was monitored for three minutes at 30° C

LDH activity was calculated using the following equation

$$\text{Activity} = [\Delta A_{rxn} / \Delta_t - \Delta A_{bkgd} / \Delta_t] \quad X \quad [V_t / 6\,22 \times l \times V_s]$$

Where ΔA_{rxn} = change in absorbance after addition of pyruvate

Δ_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

$6\,22\ l\ \text{mmol}^{-1}\text{cm}^{-1}$ = 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 $MgCl_2$ 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:

(Sample absorbance$_{15}$ – sample absorbance$_0$) – (blank absorbance$_{15}$ – 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% CO_2 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 KH_2PO_4, 0.8 mM $MgSO_4$, 1.7 mM $CaCl_2$, 16.7 mM $NaHCO_3$ 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:

hexokinase

Glucose + ATP $\longrightarrow$ glucose-6-phosphate

Glucose-6-phosphate-dehydrogenase

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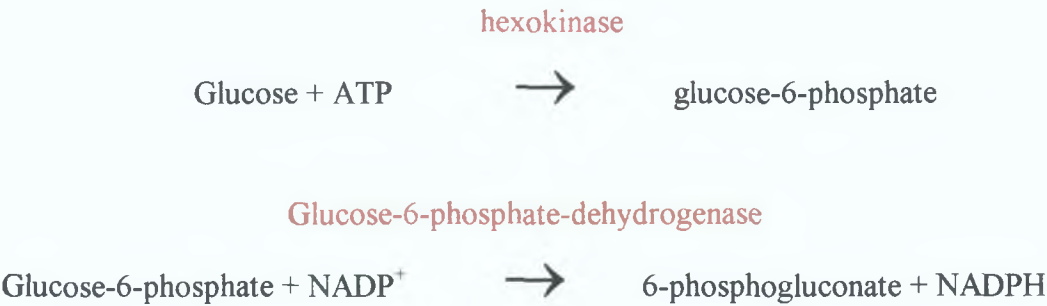
The reduction of NADP to NADPH can be monitored spectrophotometrically at 340 nm As one mole of NADP is reduced for every mole of glucose converted to 6-phosphogluconate, the change in absorbance can be directly correlated to the glucose concentration using the Beer-Lambert law which states

$$A = \varepsilon c l$$

Where A is absorbance, ε 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 umts/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

$$\frac{\Delta A \times Vt \times MW}{6\ 22 \times l \times Vs \times 1000}$$

where ΔA = the difference in absorbance at 340 nm between blank and sample,

Vt = total volume (ml)

MW = molecular weight of glucose (180 16)

6 22 = mM extinction coefficient of NADP at 340nm

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-^{3}H]leucine (5 µCi) for three hours at 37° C and 5% CO_2 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 flouride, 33 5 mM glucose-6-phosphate, 3 4 mM $NADP^+$, 2 2 mM dithiothreitol, 0 33 mM 3-hydroxy-3-methyl[3-^{14}C]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% CO_2 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 [^{3}H] mevalonolactone (2 2 x 10^4 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 H_2O_2 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 H_2O_2 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 1 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

Xanthine $\longrightarrow$ Uric acid + O_2^-

O_2^-

INT $\longrightarrow$ Formazon dye
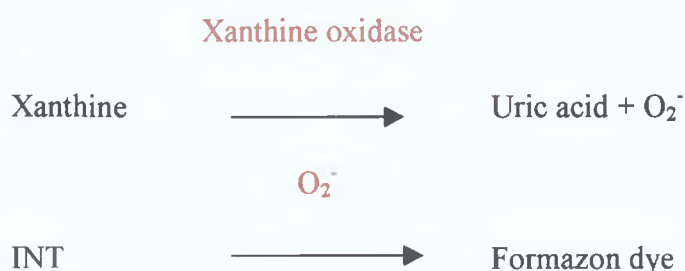
The concentration of superoxide dismutase is calculated from the rate of inhibition of this reaction.

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:

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

$$100 - \frac{(\Delta A_{sample/min} \times 100)}{(\Delta A_{blank})} = \% \text{ inhibition}$$

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

$$U/L = 8412 \times \Delta A_{340nm} / minute$$

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

91

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 1 a 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 1 b, 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
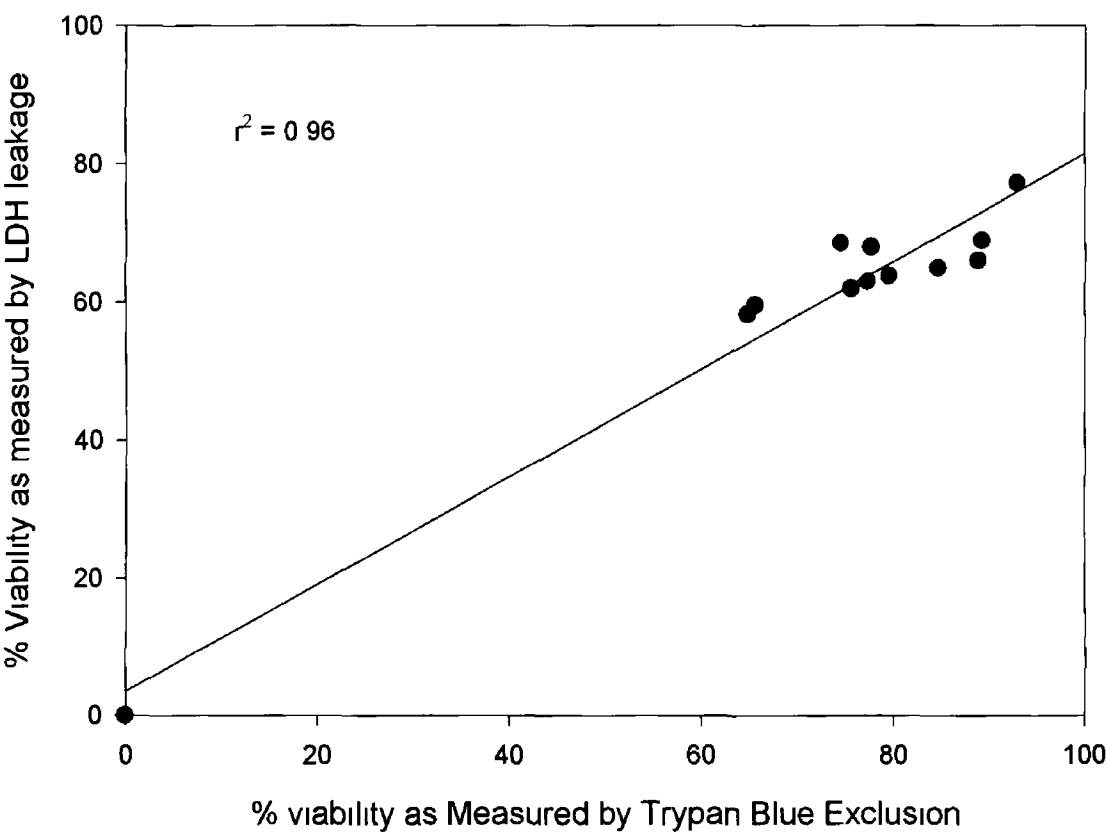
Fig 2 4 1a showing a linear correlation between LDH leakage and trypan blue exclusion as methods of determining viability The r^2 value was found to be 0 96 with 95% confidence

Fig 2.4 1 b Freshly Isolated Rat Hepatocytes
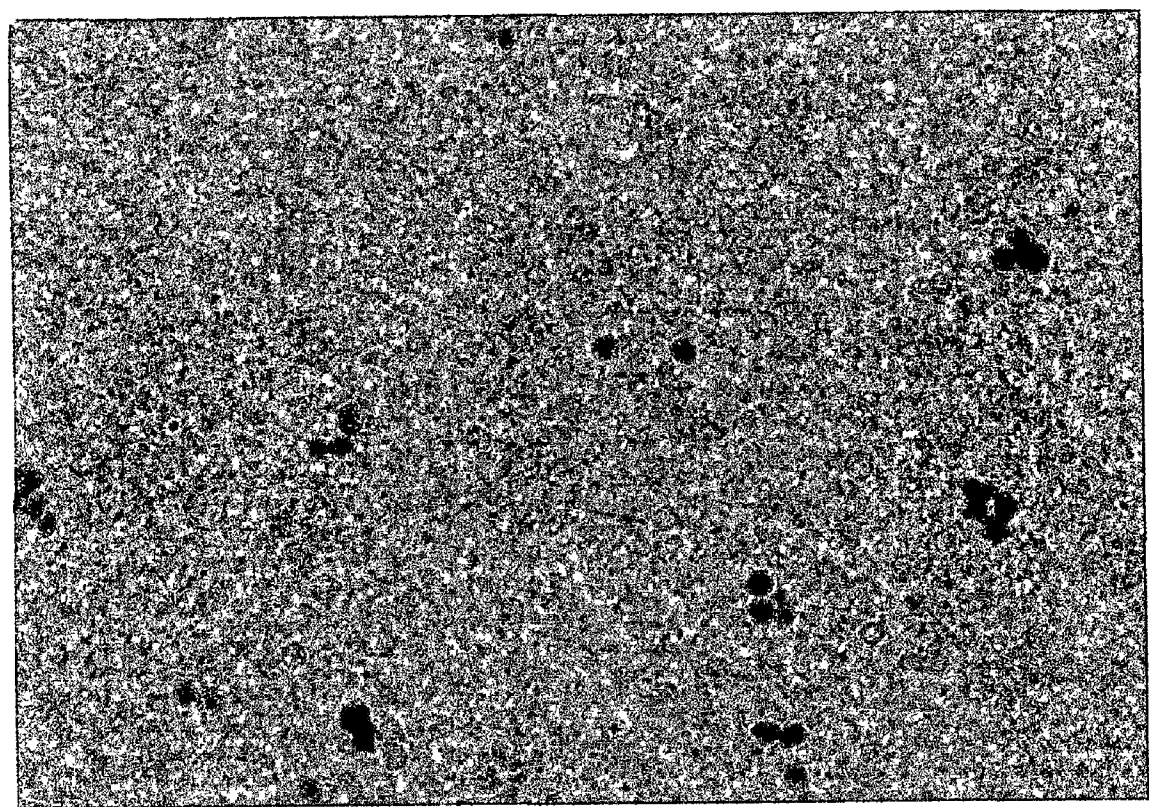
Viable cells are seen to be orange/yellow in colour and non-viable cells are seen to be

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 2 4.2a LDH Activity of 1 ml Hepatocyte Suspensions Following Sonication

Sonication Duration, seconds	LDH activity as a percentage of maximum activity observed
30	59 3 ± 3 4
60	67 7 ± 2 7
90	72 7 ± 3 3
120	60 4 ± 5 2
180	100 ± 2 1 (p<0 05)
240	25 7 ± 4 6

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 mm incubation In the presence of 0 05% Triton X100, maximum activity was released after 5 mm, while incubation for 1 mm 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

| *Incubation* | [Triton X100] | | | |
Duration, mm	*0 01%*	*0 05%*	*0 1%*	*0 15%*
1	81 4 ± 3 5	69 5 ± 3 6	100 ± 5 1	65 8 ± 5 8
3	86 1 ± 5 3	68 3 ± 4 1	73 7 ± 1 1	77 7 ± 3 2
5	79 6 ± 2 6	100 ± 4 1	67 7 ± 4 3	100 ± 4 5
10	100 ± 2 3	54 9 ± 6 1	66 5 ± 3 9	98 4 ± 2 4

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

2 4 2a 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
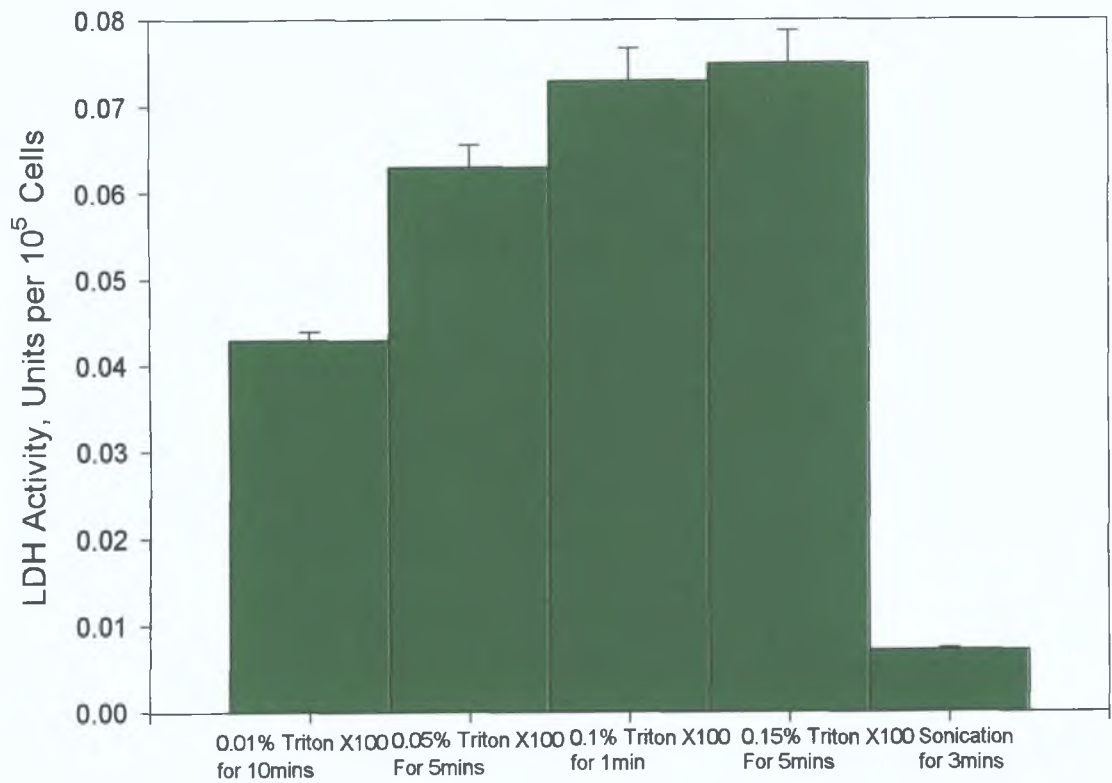
Fig. 2.4.2a showing the activity of LDH measured when 1 ml hepatocyte suspensions were lysed using different methods. For all values errors shown are SEM and n=3.

2 4 3 Protein, DNA and ATP Content

The average protein, DNA and ATP contents of freshly isolated liver cells are shown in Table 2 4 3a

Table 2.4 3a   Protein, DNA and ATP contents of freshly isolated rat hepatocytes

Protein	
mg/10⁵ cells	0 19 ± 0 015, n = 10
DNA	
μg/10⁵ cells	3 47 ± 0 37, n = 10
ATP	
mM	2 02 ± 0 43, n = 8

Errors shown are SEM

The % viability was plotted against protein content, DNA content and ATP content and the results are shown in Fig 2 4 3a, 2 4 3b and 2 4 3c 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 2 4 3d)

Fig 2 4 3a Protein Content of Cells as a Function of Viability
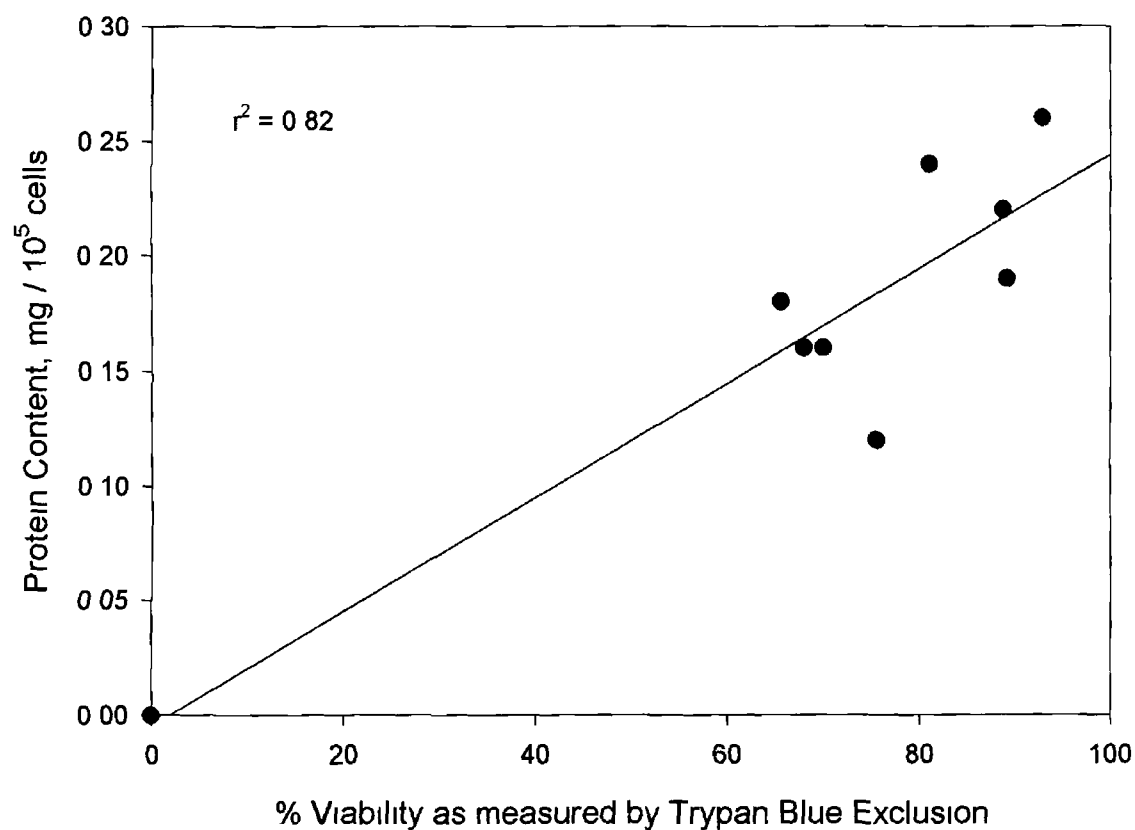
Fig 2 4 3a showing a linear correlation between protein content and % viability as measured by trypan blue exclusion The r^2 value was found to be 0 82 with 95% confidence

Fig 2 4 3b DNA Content of Cells as a Function of Viability
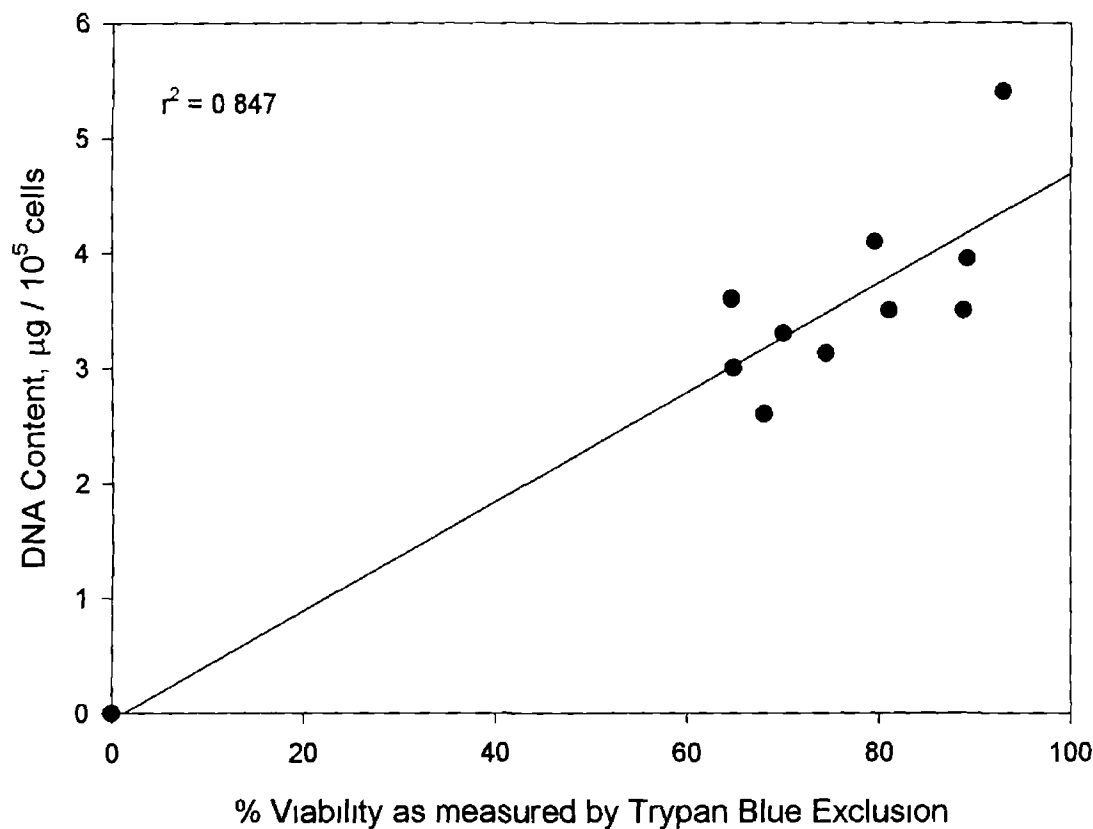
Fig 2 4 3b showing a linear correlation between DNA content and % viability
as measured by trypan blue exclusion The r^2 value was found to be
0 847 with 95% confidence

Fig 2 4 3c ATP Content of Cells as a Function of Viability
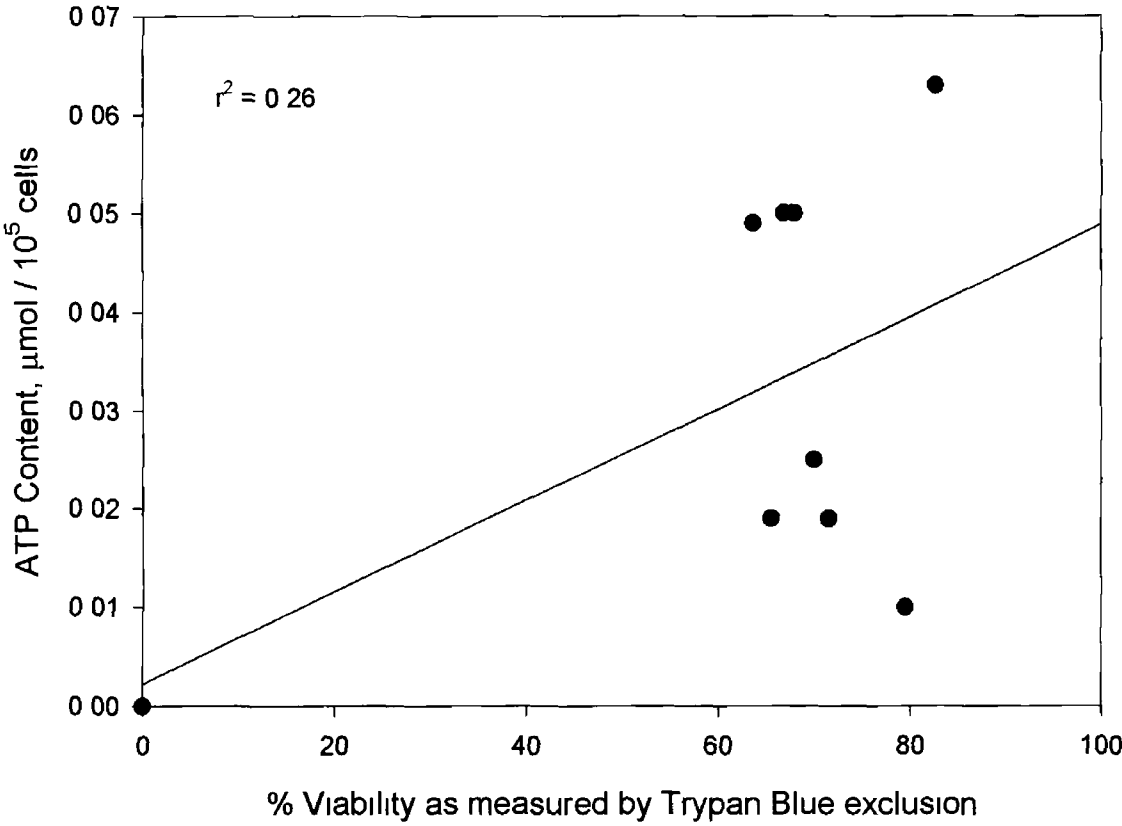
Fig 2 4 3c showing the relationship between ATP levels and % Viability No correlation is seen between the two, the r^2 value being 0 276 with 95% confidence

Fig 2 4 3d DNA Content as a Function of Protein Content
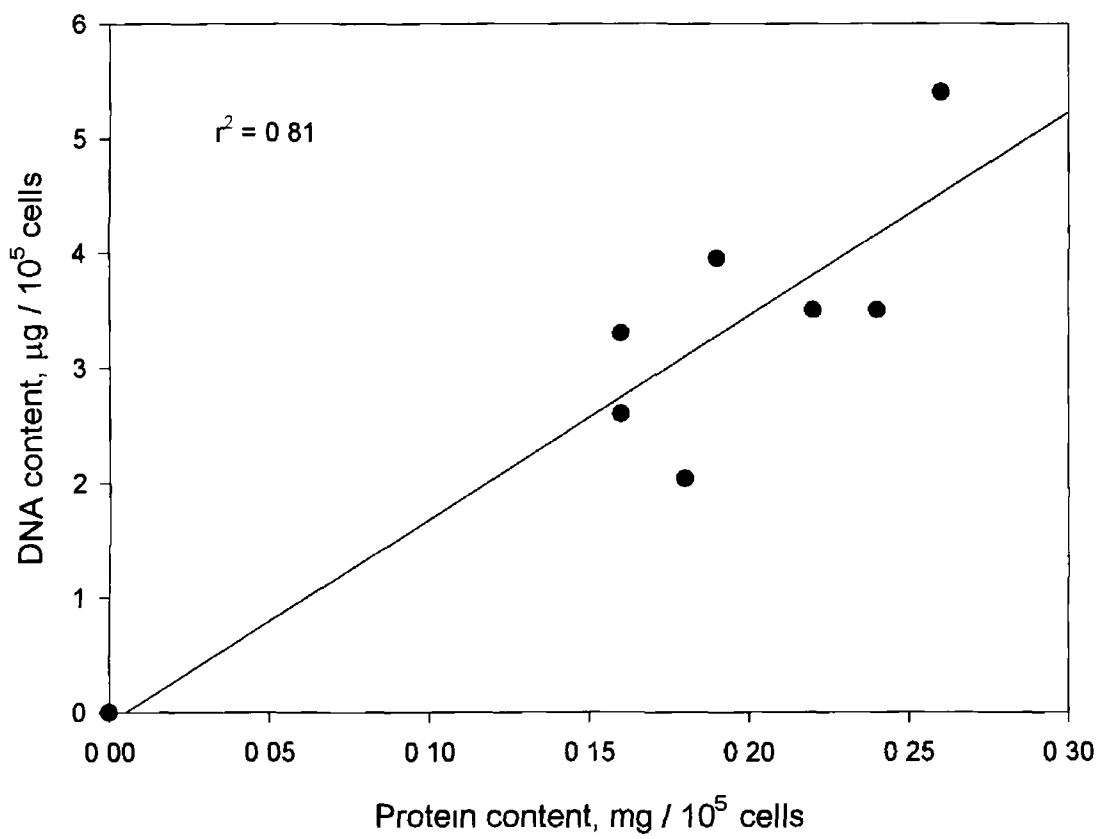
Fig 2 4 3d showing a linear correlation between protein and DNA content of hepatocytes The r^2 value was found to be 0 81 with 95% confidence

2 4 4 Gluconeogenesis, Protein Synthesis and HMG CoA reductase activity

Following incubation at $37°$ C and 5% CO_2 for 3 hours, hepatocytes in suspension in a glucose-free medium were found to produce $0\ 04 \pm 0\ 006$ mg glucose/mg protein Under similar conditions, except that glucose was supplied, hepatocytes were found to incorporate $180\ 1 \pm 11\ 7$ dpm L-[4,5-^{3}H]leucine hour^1 10^5 cells^1 The specific activity of HMG CoA reductase in suspension cultures of isolated hepatocytes was observed to be $52\ 2 \pm 9\ 5$ pmoles [^{14}C] 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

Enzyme	*Specific Activity, U/mg protein*
Catalase	64 9 $\pm$ 7 3, n=4
Superoxide dismutase	1 7 $\pm$ 0 2, n=4
Glutathione peroxidase	0 4 $\pm$ 0 02, n=5

Catalase activity was measured in Bergmeyer units which are defined as μmol [H_2O_2] 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 formazon 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 $1\,36 \pm 0\,09$ 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 s 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 polyvinylpyrrolidine 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 ± 1 5% 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 Bonis *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 Bonis *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 ± 11 65 dpm L-[4,5 – ^{3}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 ± 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 mm^{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 $52\,2 \pm 9\,5$ 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 ± 0.02 µg 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 µg 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 ± 7.3

Bergmeyer units/mg protein, a value which correlates with that published by Vasavi *et al*

(1994) of 57.05 ± 10.84 Bergmeyer units/mg protein A higher concentration of

glutathione peroxidase activity was found in this study (0.41 ± 0.02 U/mg protein)

compared to 0.095 ± 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 ± 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 ± 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 $2\ 02 \pm 0\ 46$ mM ATP and were found to incorporate $180\ 06 \pm 11\ 65$ dpm L-[4,5 – ^{3}H] leucine into protein hour^{1} per 10^{5} cells) A gluconeogenic rate of $0\ 04 \pm 0\ 006$ mg glucose production/mg protein and HMG CoA reductase activity of $52\ 2 \pm 9\ 5$ pmoles [^{14}C] 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, Covi-ox and conjugated linoleic acid (CLA) on the oxidative status of liver

CHAPTER 3

The Effect of Covi-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 (Paniangvait et al, 1995) 7-Ketocholesterol and cholestane 3β, 5α, 6β triol 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-Sallin et al, 1995, Kowale et al, 1996, Nielsen et al, 1996, Zunin et al, 1996, McCluskey et al, 1997, Shozen et al, 1997) Numerous cellular studies have implicated cholestanetriol 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 cholestanetriol 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

Fig. 3 1 1 The structure of 7-ketocholesterol and cholestanetriol
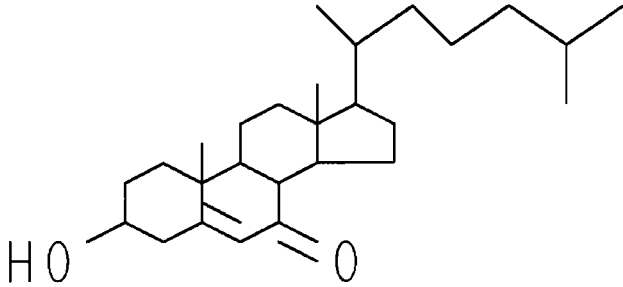
7- Ketocholesterol
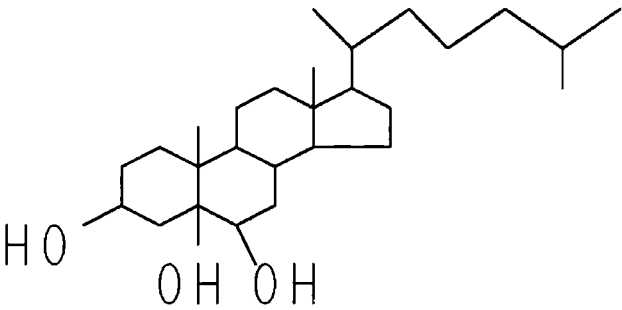
Cholestanetriol

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-^{3}H]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 β, γ and δ tocopherol and a low proportion of α 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 Covi-ox 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 ± Covi-ox 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 Covi-ox, 7-ketocholesterol, cholestanetriol and the oxysterols coincubated with Covi-ox 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 1 a 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 1 b 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 The Effect of Covi-ox +/- Oxysterols on Cell Viability

a The Effect of Covi ox on Cell Viability

b The Effect of Covi ox on Viability in
7 Ketocholesterol Treated Cells

c The Effect of Covi ox on Viability in
Cholestanetriol Treated Cells

*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 1 b) 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 800ppm 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

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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

	ATP content, *mM*
Control	0 6 ± 0 14, n = 3
Covi-ox	
100 ppm	1 1 ± 0 5, n = 3
800 ppm	1 ± 0 2, n = 3
1200 ppm	1 7 ± 0 48, n = 3
7-ketocholesterol	
100 ppm	1 2 ± 0 24, n = 4
1200 ppm	1 65 ± 0 46, n = 4
+ 800ppm Covi-ox	
100 ppm	2 25 ± 0 35*, n = 3
1200 ppm	3 35 ± 0 11*, n = 3
Cholestanetriol	
100 ppm	2 88 ± 1 4*, n = 3
1200 ppm	2 4 ± 0 9*, n = 3
+ 800ppm Covi-ox	
100 ppm	3 1 ± 0 3*, n = 3
1200 ppm	4 35 ± 0 5*, n = 3

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 ± 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)

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Fig. 3.4.2a. The Effect of Covi-ox +/- Oxysterols on Glucose Production

a. The Effect of Covi-ox on Glucose Production

b. The Effect of 7-Ketocholesterol ± 800 ppm Covi-ox on Glucose Production

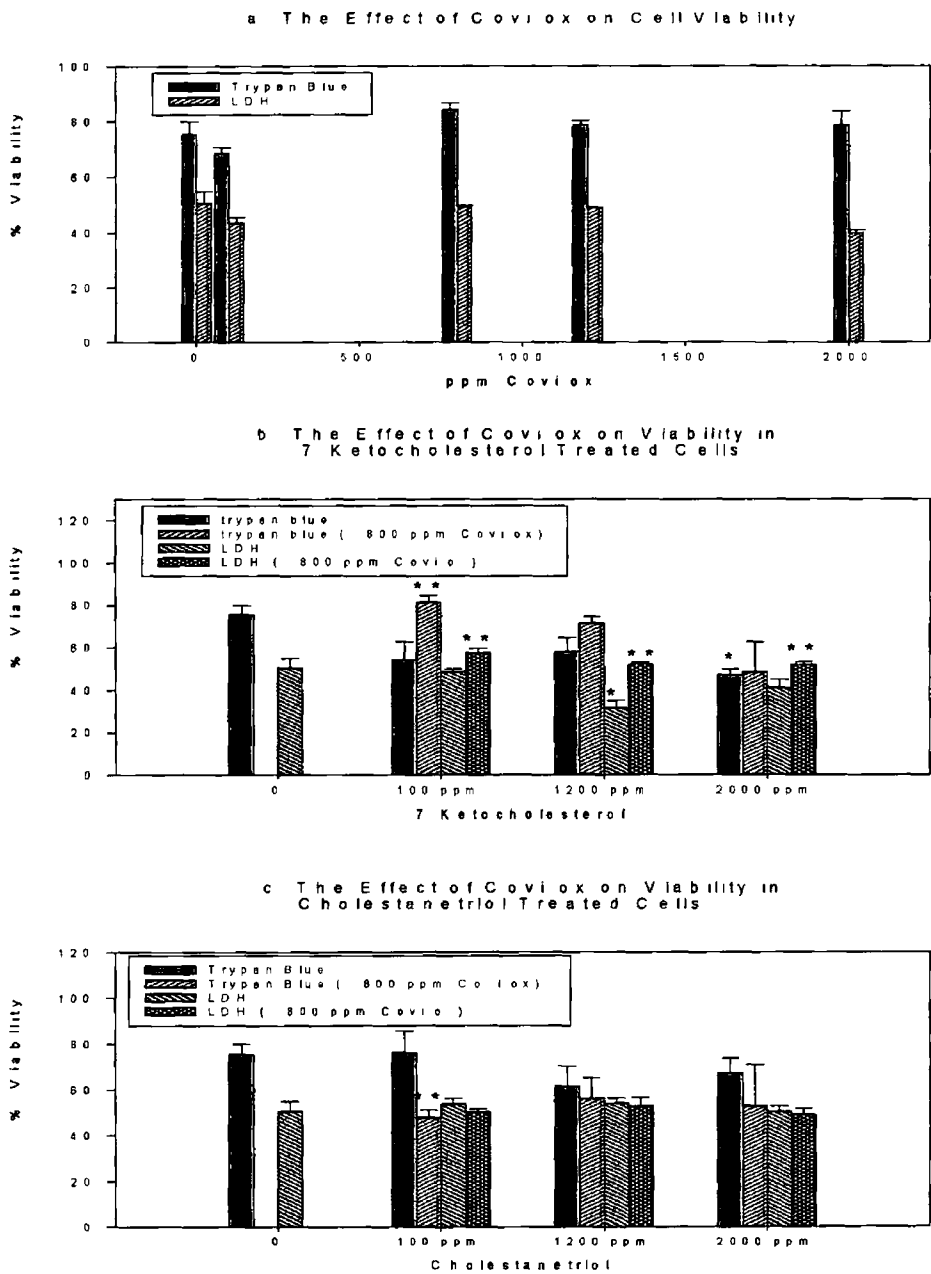c. The Effect of Cholestanetriol ± 800 ppm Covi-ox on Glucose Production
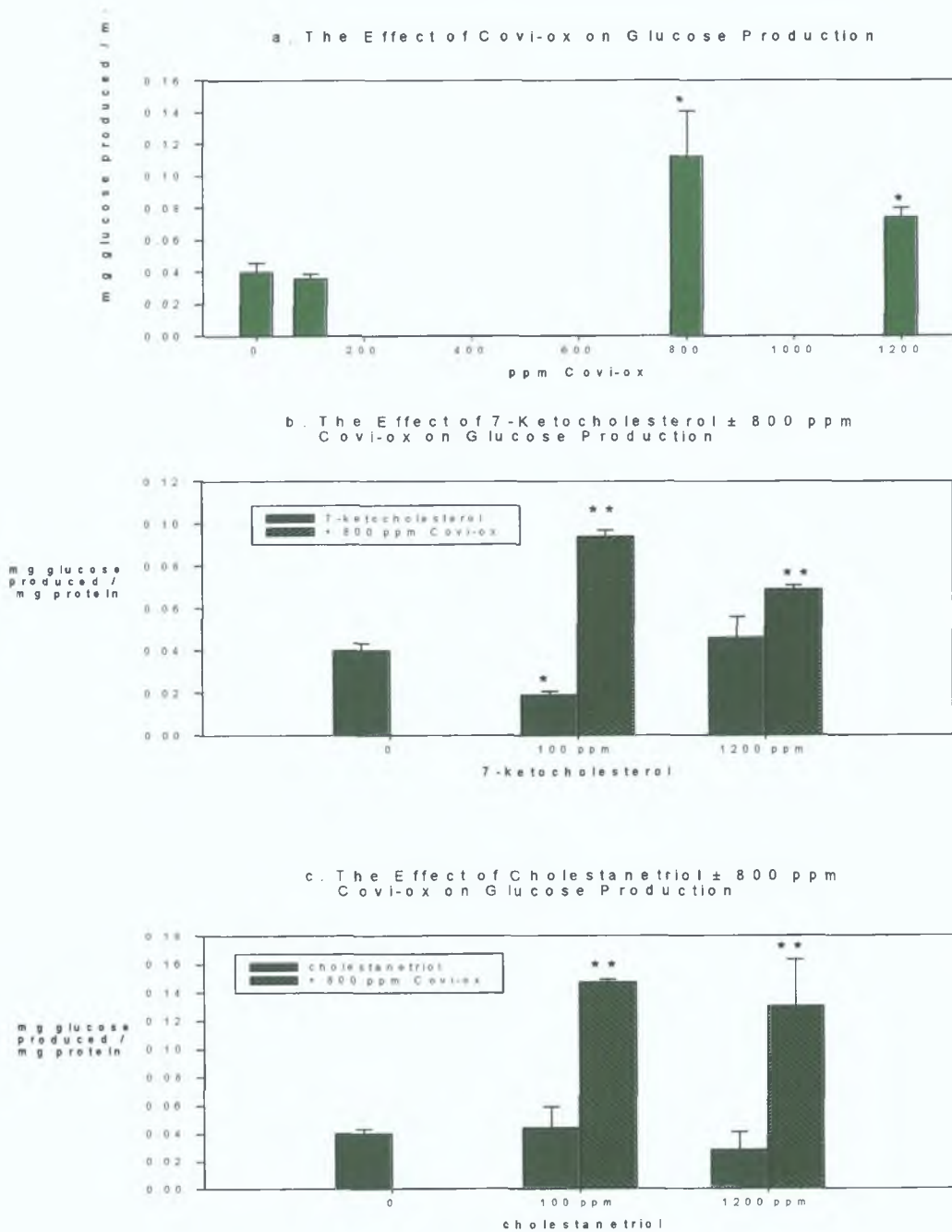
Fig. 3.4.2a showing the effect of (a) Covi-ox, (b) +/- 7-ketocholesterol or (c) +/- cholestanetriol on hepatocyte viability following incubation for 3 hours. *indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) to the value obtained in the absence of Covi-ox. For 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.
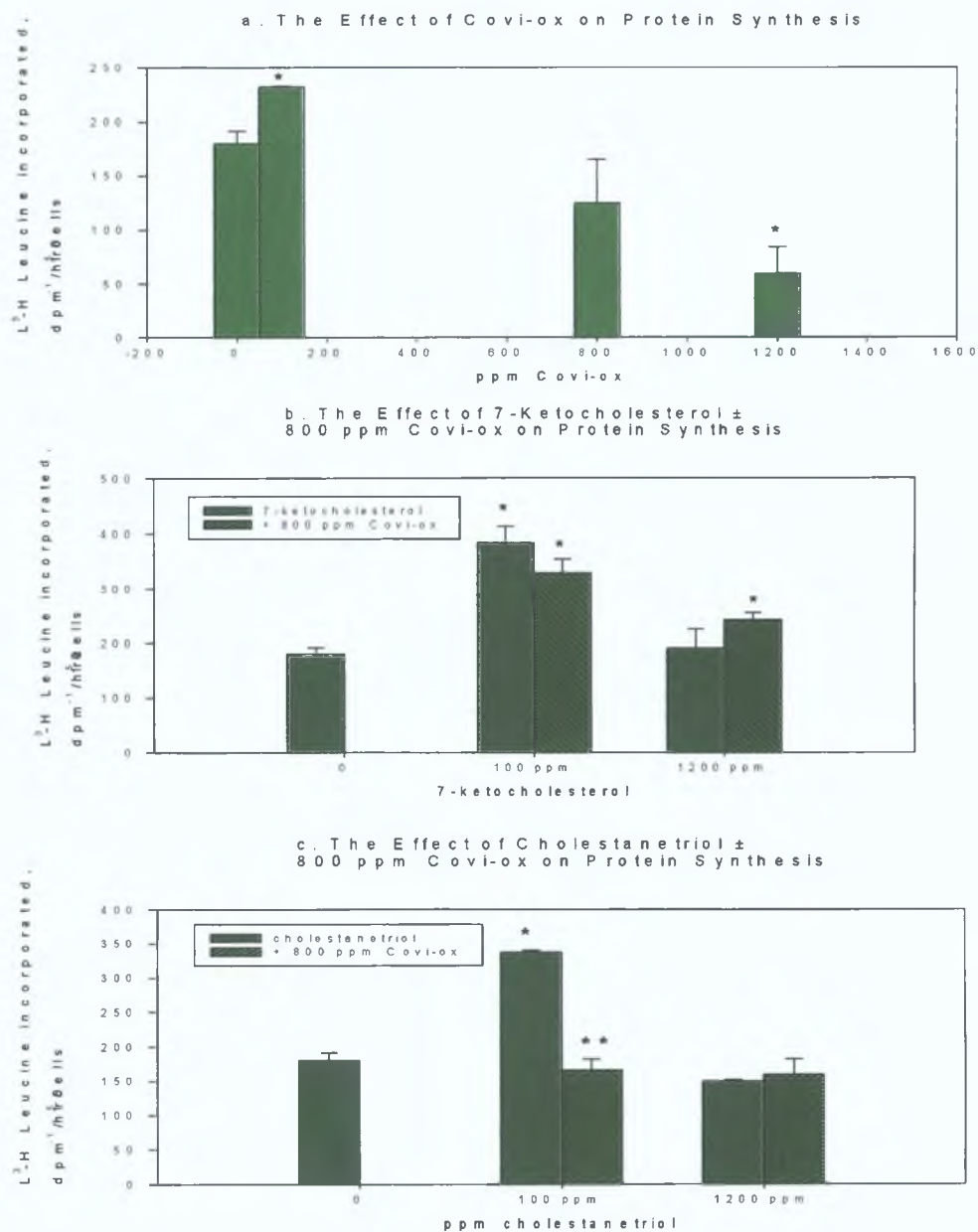
*Fig 3.4.2b showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) or +/- cholestanetriol (c) on protein synthesis as measured by the incorporation of L-^3H leucine. For all values n=3, errors shown are SEM. *indicates a significant (p<0.05 difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox.*

3 4 3 Antioxidant Enzyme Levels

SOD Activity

Incubation with increasing concentrations of Covi-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 Covi-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 Covi-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 Covi-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 Covi-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 Covi-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

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Fig. 3.4.3a The Effect of Covi-ox +/-Oxysterols on SOD Activity

a. The Effect of Covi-ox on SOD Activity

c. The Effect of Covi-ox on SOD Activity in
7-Ketocholesterol Treated Cells

b. The Effect of Covi-ox on SOD Activity in
Cholestanetriol Treated Cells

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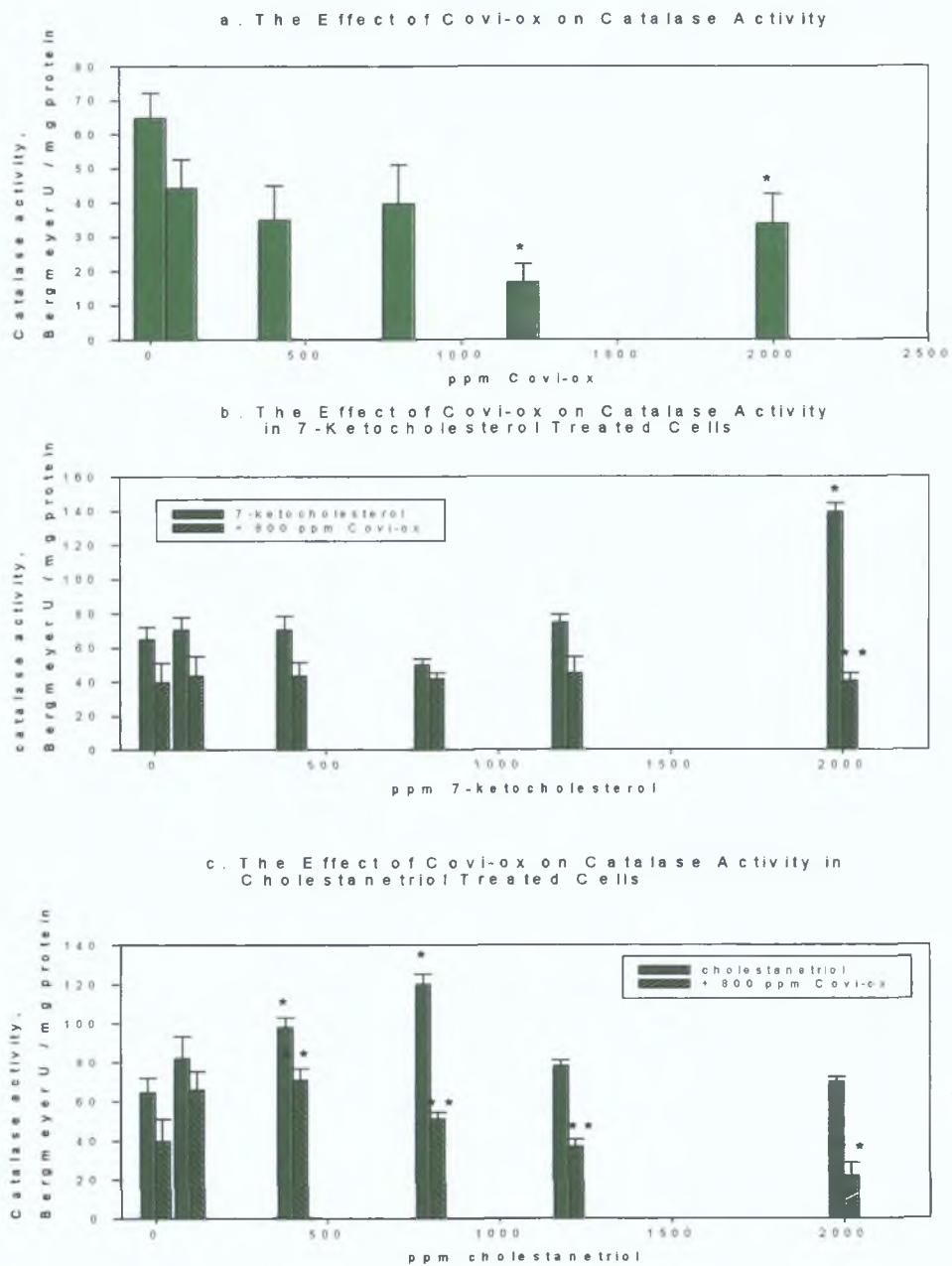
Fig. 3.4.3b showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on Catalase activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence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

3 4 3 b(b)) 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 cholestantriol 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. 3.4.3.c The Effect of Covi-ox +/- Oxysterols on GPx Activity

a . T h e   E f f e c t   o f   C o v i - o x   o n   G P x   A c t i v i t y

b .   T h e   E f f e c t   o f   C o v i - o x   o n   G P x   A c t i v i t y   i n
7 - K e t o c h o l e s t e r o l   T r e a t e d   C e l l s

c .   T h e   E f f e c t   o f   C o v i - o x   o n   G P x   A c t i v i t y   i n
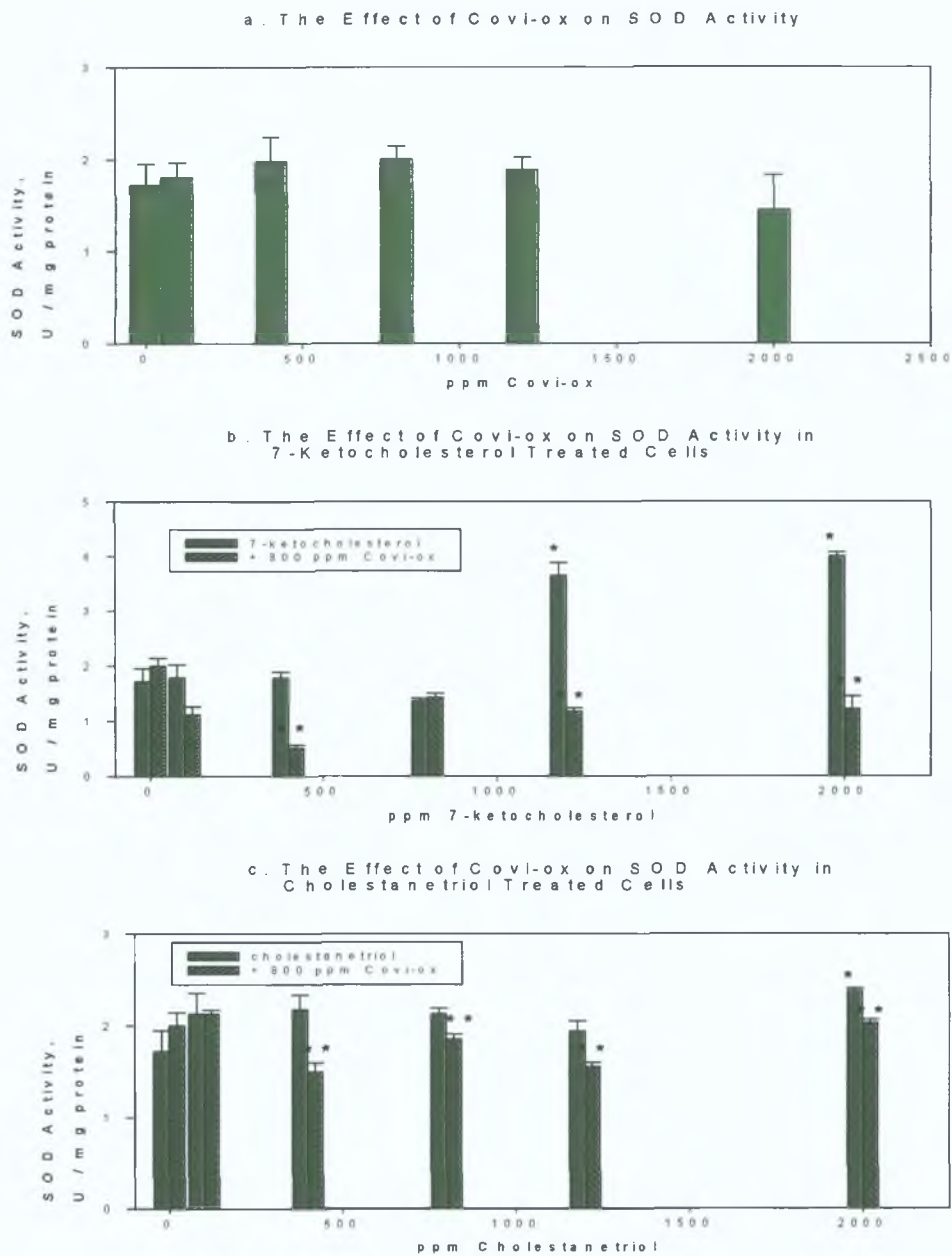C h o l e s t a n e t r i o l   T r e a t e d   C e l l s
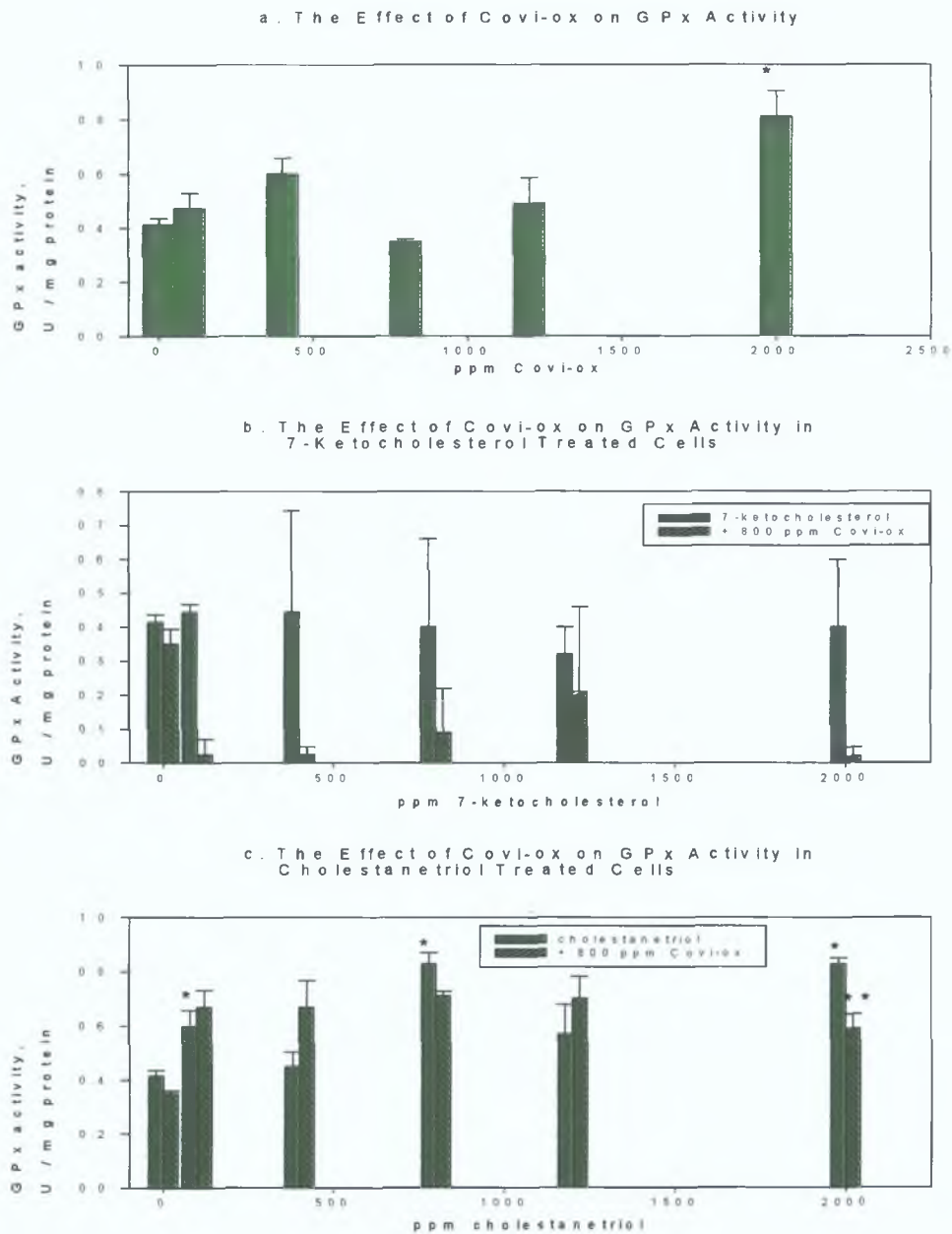
*Fig. 3.4.3c showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on GPx activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox*

level In the presence of 2000 ppm cholestanetriol, Covi-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(c))

3 4 4 Lipid Peroxidation

Neither Covi-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 Covi-ox than in its absence (Fig 3 4 4 (b),(c)) Covi-ox had negligible effects at higher concentrations of oxysterol

Fig. 3.4.4 The Effect of Covi-ox +/- Oxysterols on Lipid Peroxidation

a. The Effect of Covi-ox on Lipid Peroxidation

b. The Effect of Covi-ox on Lipid Peroxidation in 7-Ketocholesterol Treated Cells

c. The Effect of Covi-ox on Lipid Peroxidation in Cholestanetriol Treated Cells
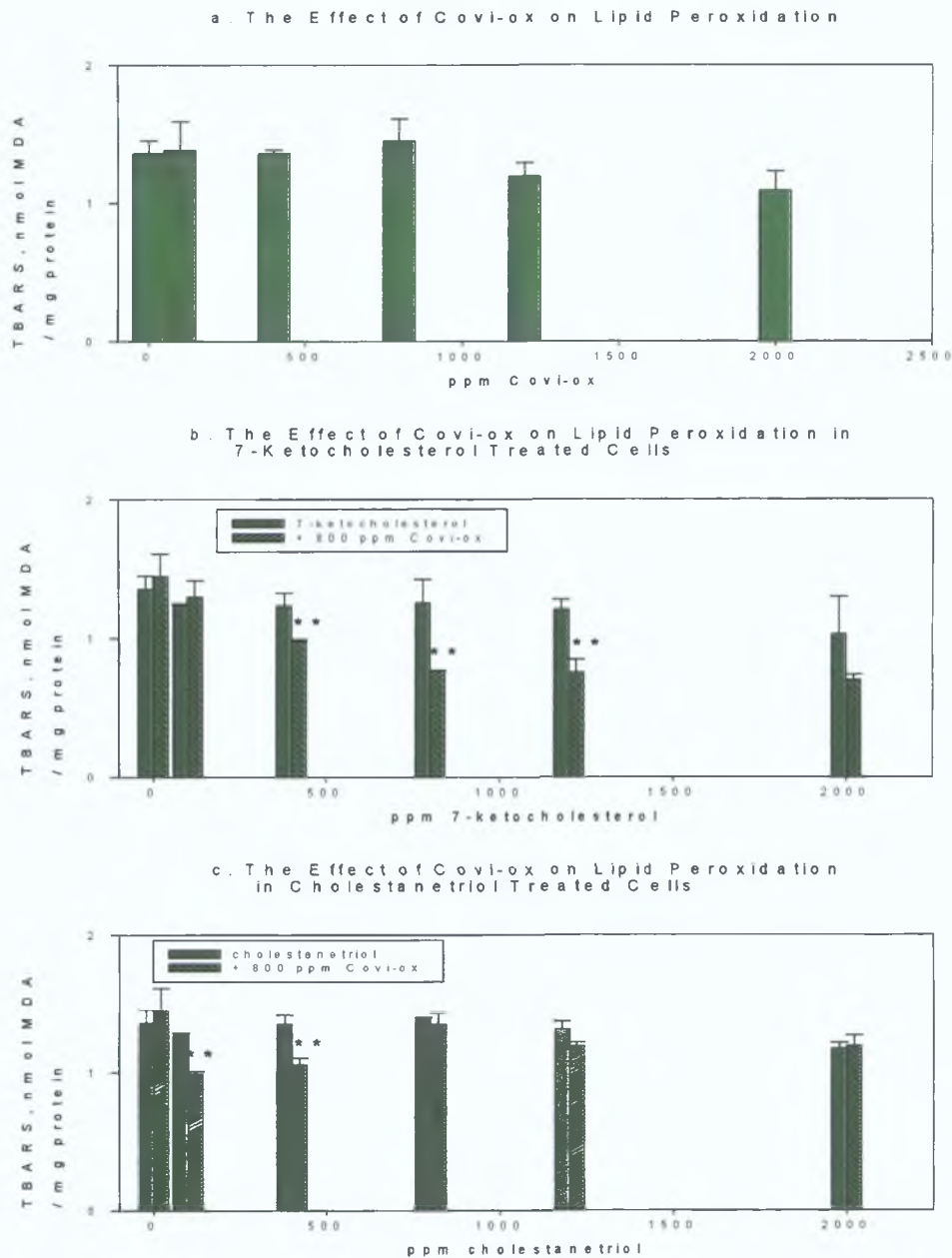
Fig. 3.4.4 showing the effect of Covi-ox (a), +/- 7-ketocholesterol (b) and +/- cholestanetriol (c) on lipid peroxidation as measured by TBARS. For all values n=3, errors shown are SEM. **Indicates a significant (p<0.05) difference to the value obtained in the absence of Covi-ox

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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*

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(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 Coincubation 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 cholestanetriol 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

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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 Covi-ox

3.6 Summary

Suspension cultures of isolated rat hepatocytes were used to investigate whether 7-ketocholesterol and cholestane3β, 5α, 6β triol 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 cholestane 3β, 5α, 6β triol Covi-ox, 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 fibrosolvens* 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 in to 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 methylnitrosurea (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 Pariza *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 [^{3}H] mevalonolactone (33 Ci/mmol), 3-hydroxy-3-methyl[3-^{14}C]glutaryl-CoA (45 6 mCi/mmol), L-[4,5-^{3}H]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 cholestanetriol 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% CO_2 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 cholestanetriol, 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

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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 methanolysis 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, 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 i d , 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 cholestanetriol, 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
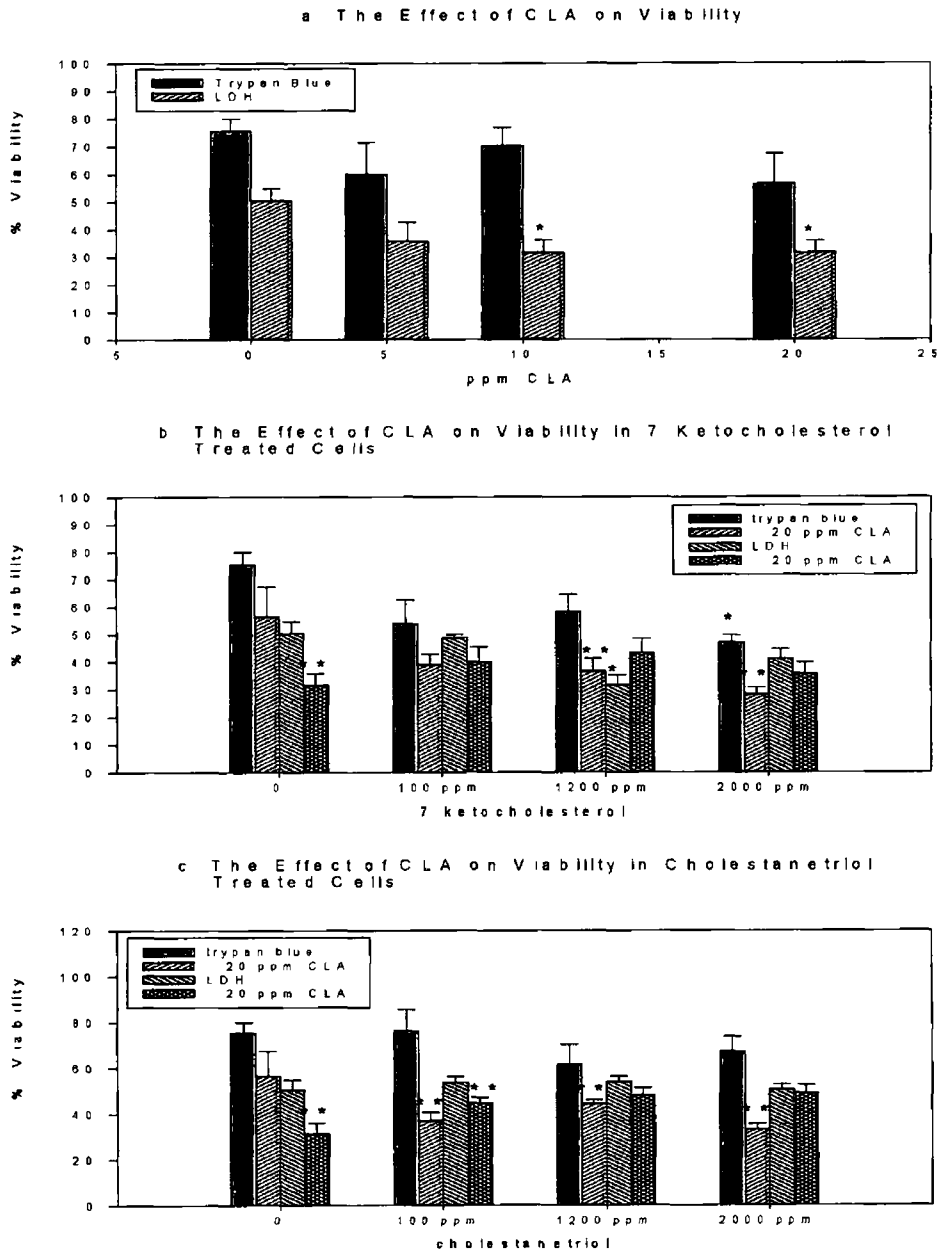
Fig 4 4 1 showing the effect of (a) CLA (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on hepatocyte viability following a three hour incubation For all values n=3, errors shown are SEM *Indicates a significant (p<0 05) difference to control, **indicates a significant (p<0 05) difference to the value obtained in the absence of CLA

4 4 2 CLA Uptake into Liver Lipid Fractions

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, linolemc and *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, *trans* C18 1 acid, linoleic acid, γlinolenic 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 *cis*-9, *trans*-11 and *trans*-10, *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 Coincubation 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 γLinolenic 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 γlinolenic 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
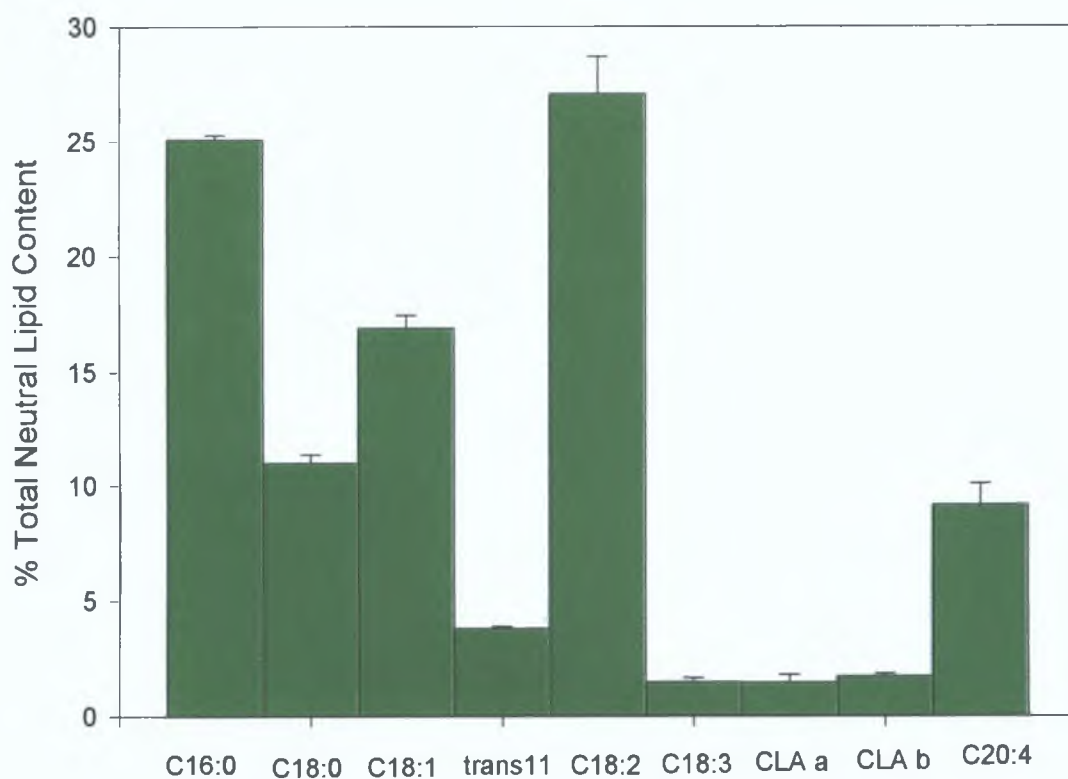
Fig 4.4.2a showing the neutral lipid profile of control hepatocytes following a three hour incubation. For all values shown n=3 and errors shown are SEM.

C16:0 = palmitic acid   *C18:0= stearic acid*   *C18:1= oleic acid*

trans11= trans-11-C18:2   *C18:2= linoleic acid*   *C18:3= γlinolenic 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
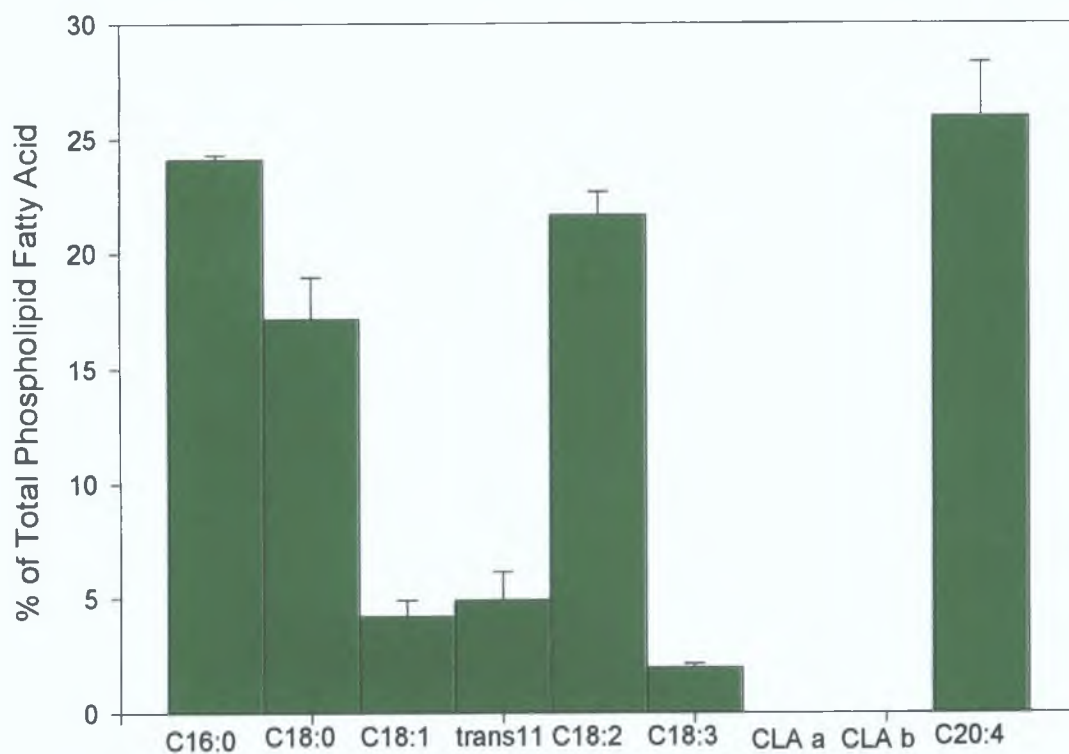
Fig 4.4.2b showing the fatty acid profile of the phospholipid fraction of liver cells following a three hour incubation. For all values n=3, errors shown are SEM.

C16:0 = palmitic acid   C18:0 - stearic acid   C18:1 - oleic acid

trans11 = trans-11-C18:1   C18:2 = linoleic acid   C18:3 = γlinoleic 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 4.2a The effect of CLA in the presence and absence of 7-ketocholesterol and cholestanetriol on the fatty acid composition of the neutral lipid fraction of rat hepatocytes

Fatty Acid	Control	20ppm CLA	+ 800ppm 7keto	+1200ppm 7keto	+2000ppm 7keto	+800ppm triol	+1200ppm triol	+2000ppm triol
16 0	25 1±0 2	24 9±1 5	26 4±0 7	25 9±1	28±1 2	26 3±0 9	27 6±0 8	25 6±1 1
18 0	10 9±0 4	9 2±0 2	9±0 8	9 9±1	8 4±1	7 9±0 3*	6 7±0 7*	7 2±0 04*
18 1	16 9±0 6	15 6±0 8	15 1±0 4	15 2±0 8	15 4±0 7	13 7±0 1	13 8±0 7	11 9±0 8*
18 1 trans 11	3 8±0 1	3 8±0 2	3 6±0 04	3 7±0 2	3 8±0 3	3 6±0 05	3 4±0 4	3 7±0 4
18 2	27 1±1 7	27 6±1 2	28 3±0 2	26 6±1 7	27 6±0 7	27 2±0 6	28 7±1 1	30 7±0 9
18 3	1 5±0 2	1 4±0 3	1 4±0 1	1 5±0 1	1 5±0 1	1 1±0 04	1 6±0 2	1 3±0 04
18 2 cis 12	1 4±0 4'	3 9±0 6^c	3 36±0 1^c	4 32±0 5^c	2 2±0 5	3 5±0 2^c	4 2±0 3^c	4 2±0 3^c
18 2 cis 9	1 7±0 1	4 1±0 4^c	3 9±0 2^c	4 2±0 2^c	2 3±0 4*	3 4±0 1^c	3 4±0 2^c	3 2±0 06^c
20 4	9 2±1	9 1±1 1	10 4±0 5	9 6±0 8	10 3±0 6	10 3±0 8	10 6±1 2	11 9±0 9

*Values are means ± SEM, n=3 Values are expressed as percentage total fatty acid ^{c} 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*

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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

Fatty Acid	Control	20ppm CLA	+ 800ppm 7keto	+1200ppm 7keto	+2000ppm 7keto	+800ppm triol	+1200ppm triol	+2000ppm triol
16 0	24 1±0 2	25 5±1 3	24 9±0 2	25 5±0 6	25 6±0 4	26 7±0 9	24 9±1 2	25 6±0 6
18 0	17 2±1 8	22±1 5	19 3±0 05	18 3±0 3	18 9±0 3	16 5±0 7*	18 9±0 2	16 8±1 7
18 1	4 2±0 7	6 7±0 6^c	4 7±0 2*	4 7±0 14*	4 5±0 2*	4 2±0 2*	4 8±0 2*	4 7±0 03*
18 1 trans 11	4 9±1 2	6 4±0 8	4 7±0 2*	5 2±0 3	5 5±0 4	4 7±0 3	4 9±0 2	4 8±0 04*
18 2	21 7±1	18 1±1 6	22 6±0 2*	21 8±0 7	23 6±0 5*	22 7±0 2*	22 9±0 4*	22 6±0 3*
18 3	2±0 2	0^c	14±0 02*^{c}	16±0 03*^{c}	13±0 02*^{c}	1±0 01*^{c}	14±0 02*^{c}	17±0 02*^{c}
18 2 cis 12	0	0 7±0 1^c	0 2±0 02*^{c}	0 2±0 01*^{c}	0 2±0 02*^{c}	0 2±.004*^{c}	0 2±0 01*^{c}	0 1±0 01*^{c}
18 2 cis 9	0	0 8±0 1^c	0 4±0 05*^{c}	0 4±0 03*^{c}	0 35±02*^{c}	0 3±0 02*^{c}	0 3±0 03*^{c}	0 4±0 04*^{c}
20 4	26±2 4	20 7±1 7	22 9±0 1	23 7±0 3	23 7±0 5	24±0 2	23 7±0 3	25 2±1 1

*Values are means ± SEM, n=3 Values are expressed as percentage total fatty acid ^{c} 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*

4 4 3 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

	ATP Content / mM	
	CLA/oxysterol alone	In the presence of 20 ppm CLA
Control	0 6 ± 0 14, n = 3	
CLA		
5ppm	0 53 ± 0 12, n = 3	
10ppm	3 45 ± 2 12, n = 3	
20ppm	0 6 ± 0 06, n = 3	
7Ketocholesterol		
100ppm	1 2 ± 0 24, n = 4	1 4 ± 0 57, n = 3
1200ppm	1 65 ± 0 46, n = 4	2 2 ± 0 68*, n = 3
Cholestanetriol		
100ppm	2 88 ± 1 4*, n = 3	5 95 ± 2 9*, n = 3
1200ppm	2 4 ± 0 95*, n = 3	3 ± 0 9*, n = 3

*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(b) 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 3 c 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

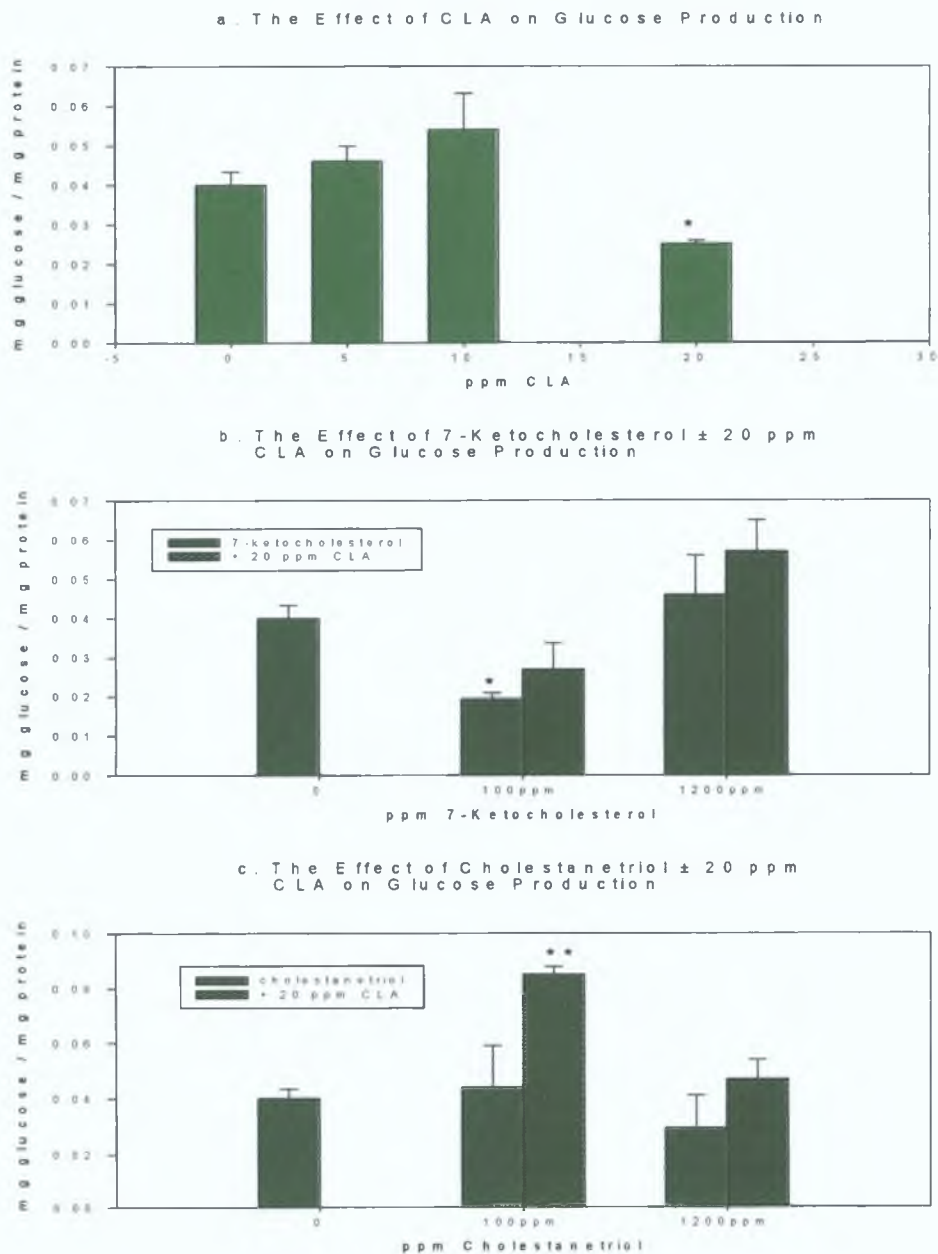**Fig. 4.4.3b The Effect Of CLA +/- Oxysterols on Protein Synthesis**

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.

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Fig. 4.4.3c The Effect of CLA in the presence and absence of 7-Ketocholesterol and Cholestanetriol on Cholesterol Synthesis
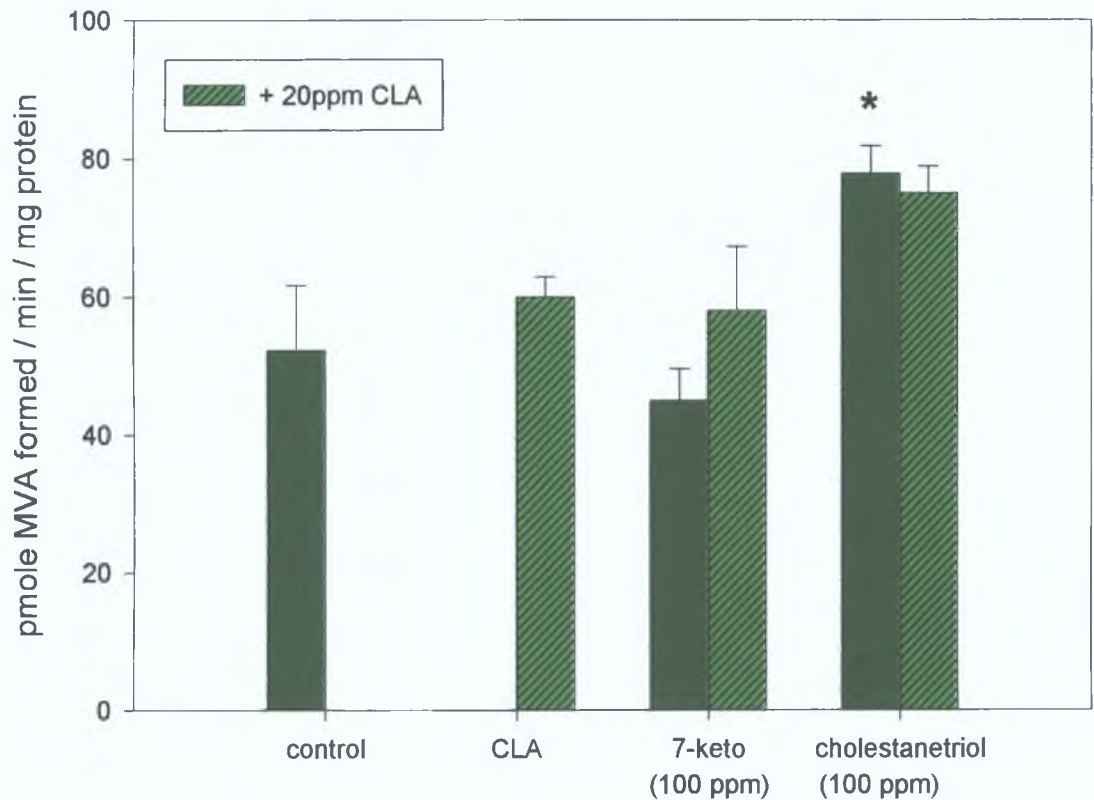
Fig 4.4.3c showing the effect of CLA and of oxysterols in the presence and absence of 20 ppm CLA on cholesterol synthesis as measured by the activity of expressed HMG CoA reductase activity. For all values n=4, errors shown are SEM and * indicates a significant (p<0.05) difference to control cells.

4 4 4 The Effect of CLA on Antioxidant Enzymes

Superoxide dismutase

The effect of CLA on SOD activity is shown in Fig 4 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 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 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 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 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 4 f shows the effect of CLA on cholestanetriol-treated cells In cells incubated with

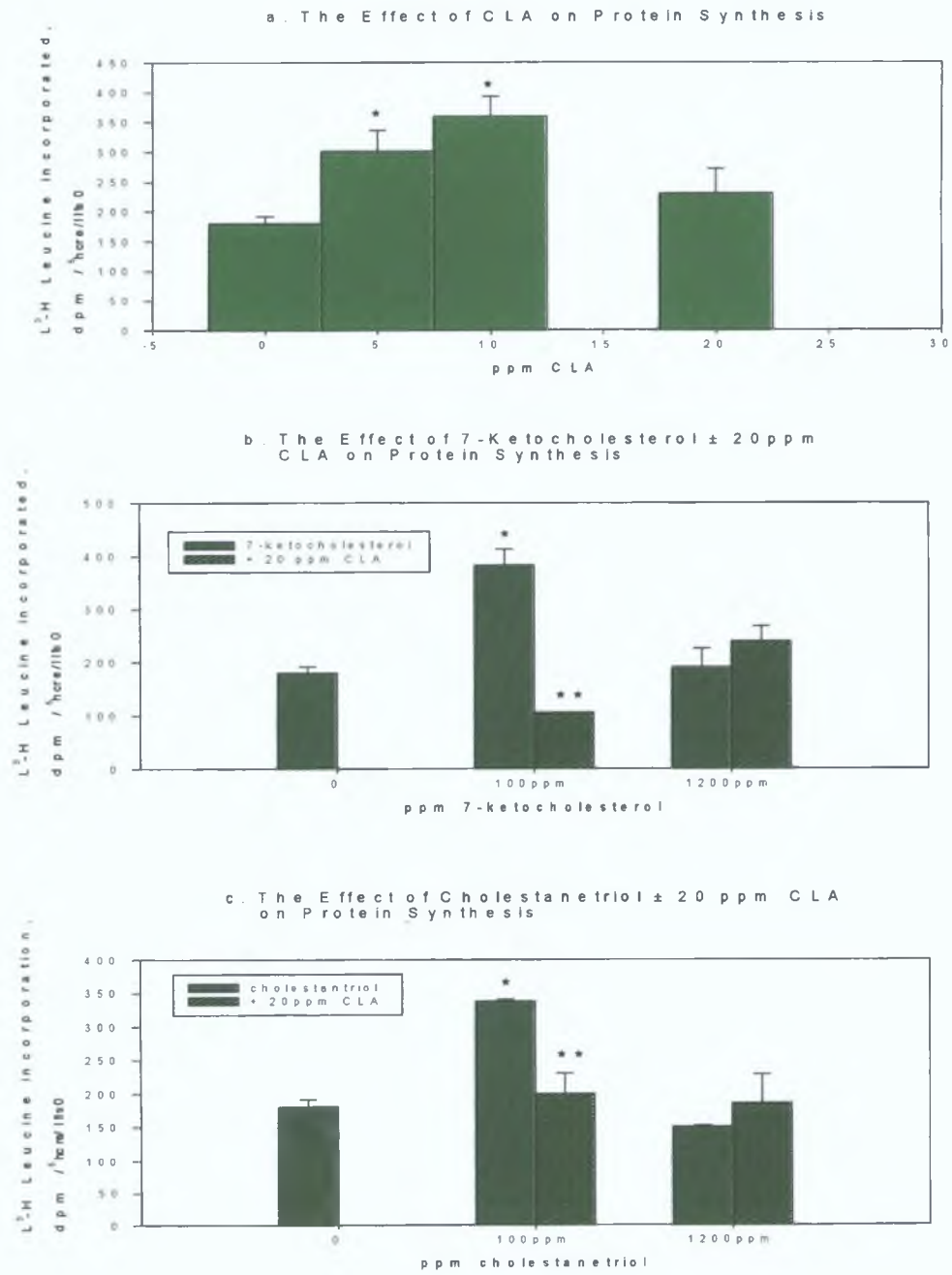## Fig. 4.4.4a The Effect of CLA +/- Oxysterols on SOD Activity

*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 coincubation with 20 ppm CLA relative to treatment with 7-ketocholesterol alone (Fig 4 4 5(b)) A

Fig. 4.4.4c The Effect of CLA +/- Oxysterols on GPx Activity

a. The Effect of CLA on GPx Activity

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

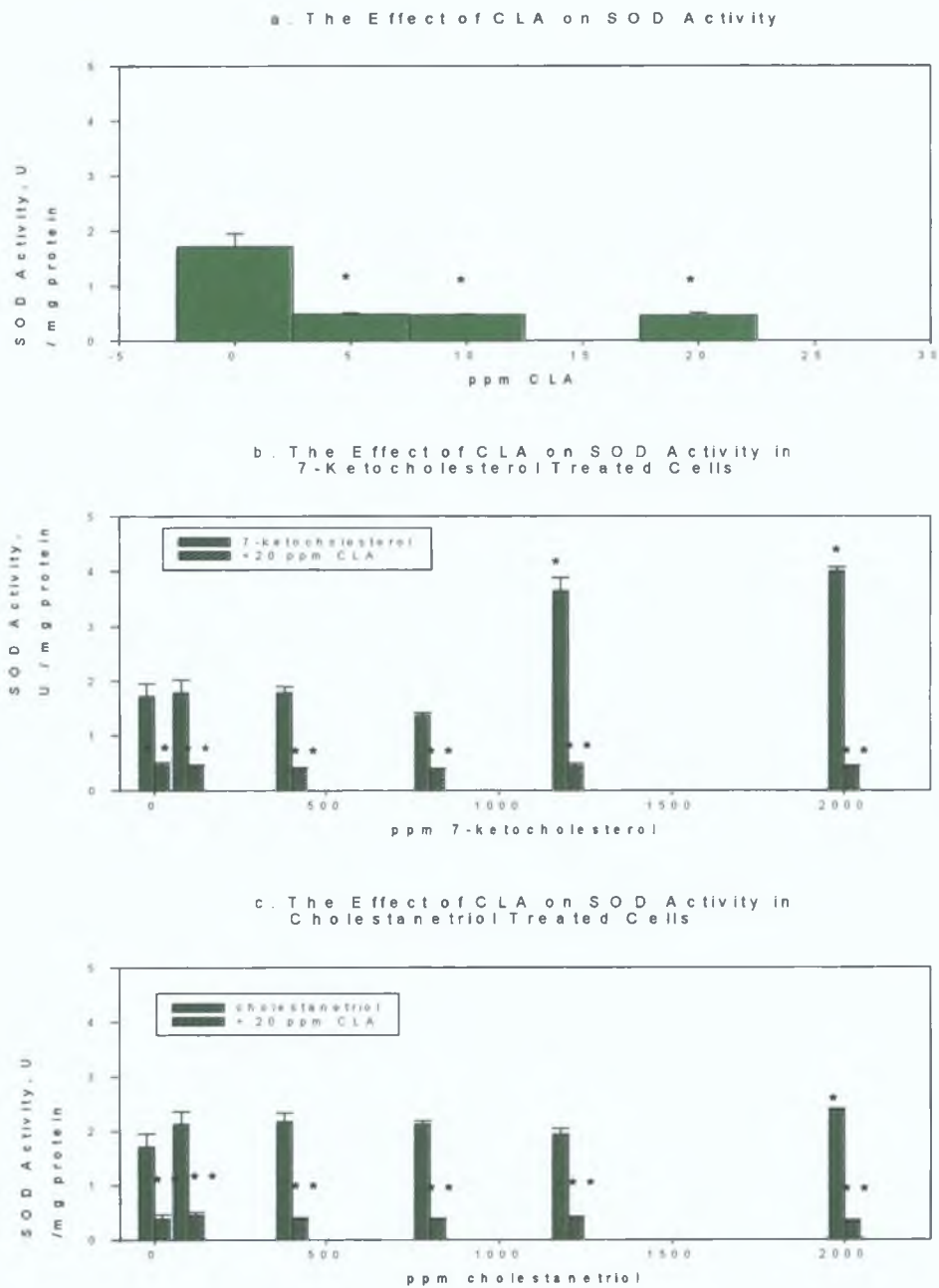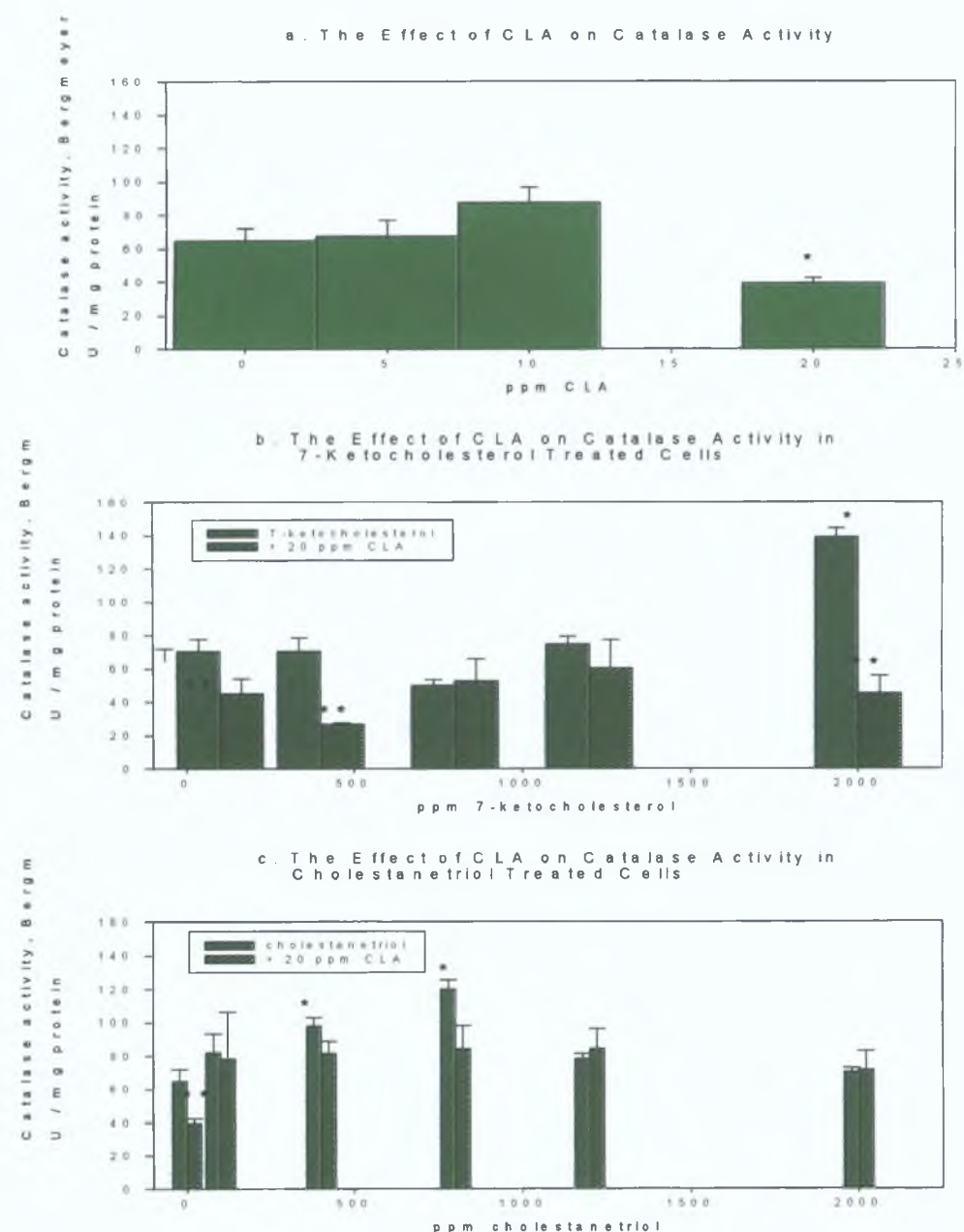c. The Effect of CLA on GPx Activity in
Cholestanetriol Treated Cells
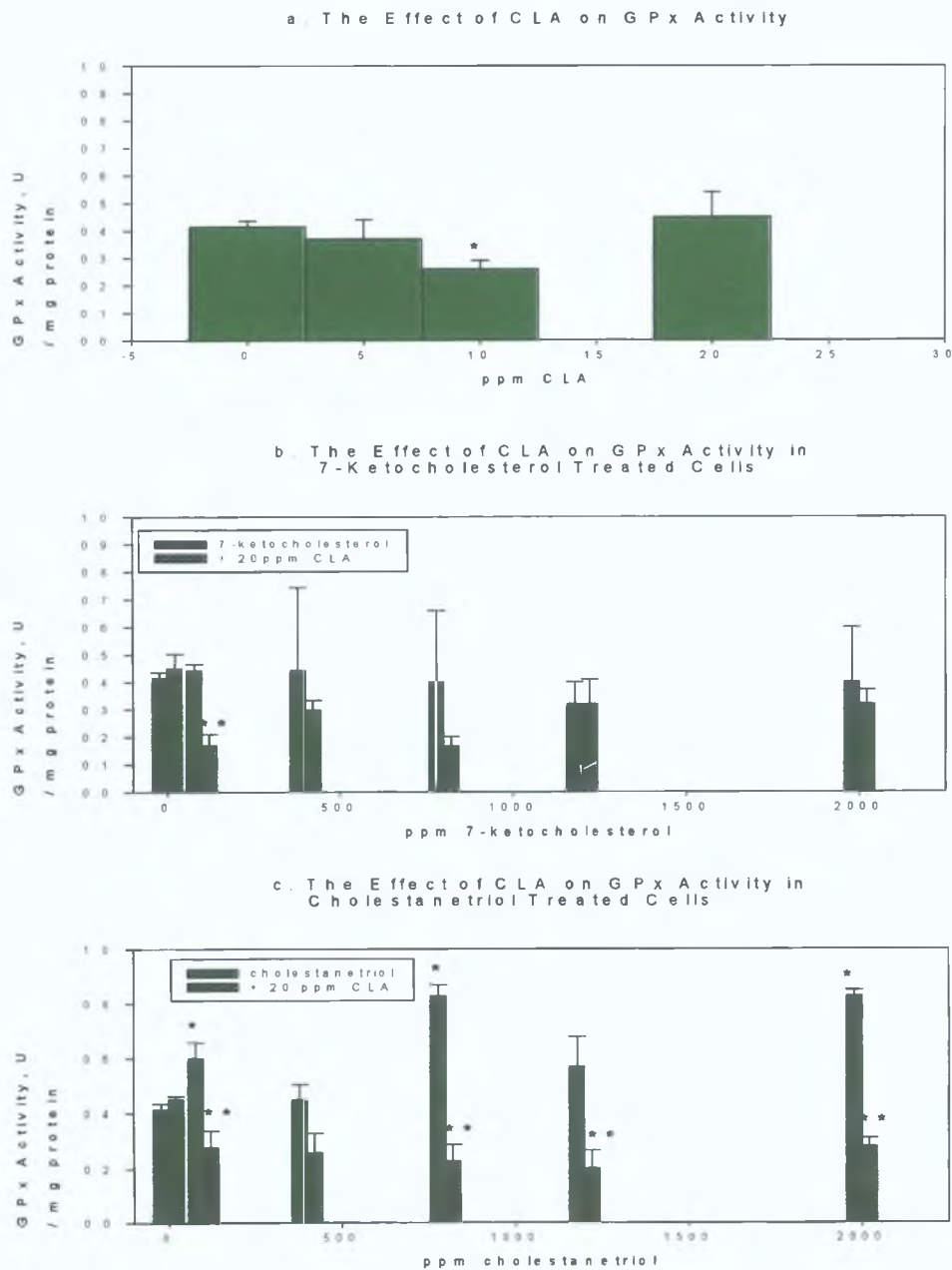
Fig. 4.4.4c showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on GPx activity. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained in the absence of CLA.

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

a. The Effect of CLA on Lipid Peroxidation

b. The Effect of CLA on Lipid Peroxidation in 7-Ketocholesterol Treated Cells

c. The Effect of CLA on Lipid Peroxidation in Cholestanetriol Treated Cells
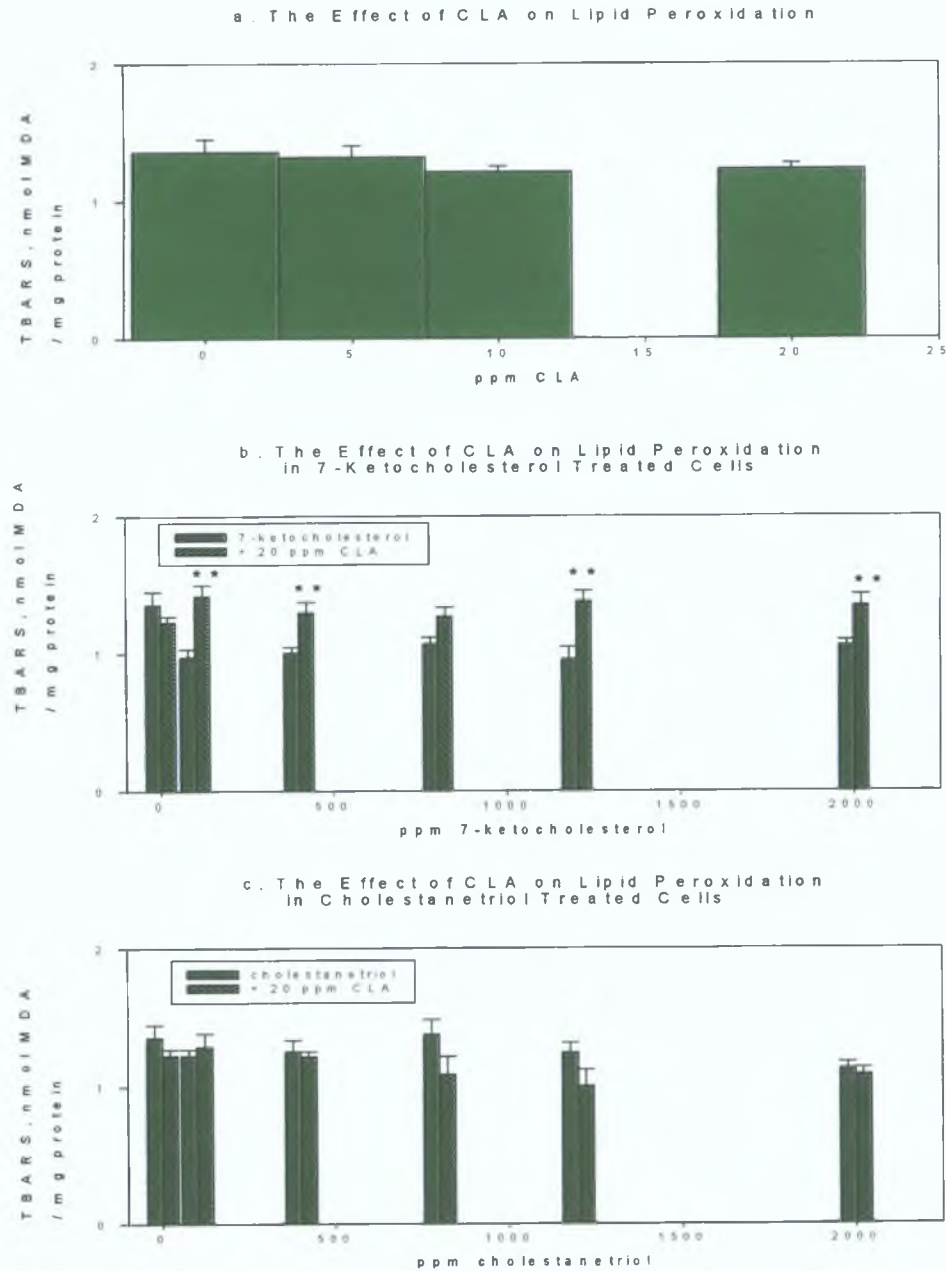
*Fig. 4.4.5 showing the effect of (a) CLA, (b) +/- 7-ketocholesterol and (c) +/- cholestanetriol on Lipid Peroxidation as measured by TBARS. For all values n=3, errors shown are SEM. *Indicates a significant (p<0.05) difference to control, **indicates a significant (p<0.05) difference to the value obtained 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 lever 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, cycloxygenase and lipoxygenase enzyme families. Although liver paranchymal 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 *in vivo* animal systems and finally to *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 *in vivo* making hepatocytes the cell type of choice for *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 *et al* (1984) compared three different types of collagenase These were collagenase type IV from *Clostridium histolyticum*, hepatocyte isolation grade, supplied by Sigma Ltd , St Louis, Mo, collagenase no 100502 isolated from *C histolyticum* supplied by ICN Pharmaceuticals Inc , Cleveland, Ohio and collagenase/dispase (dispase is a neutral protease) isolated from *Achromobacter iophagus* and *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 µU/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 clostripain, 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 iophagus 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 iophagus 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 iophagus/ 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

Collagenase	Cell Yield, per g wet weight	% Viability
Collagenase IA	$6\ 62 \pm 1\ 93 \times 10^7$	$71\ 5 \pm 3\ 8$
Collagenase IV	$2\ 04 \pm 11 \times 10^6$	$55 \pm 10\ 6$
Collagenase/dispase	$4\ 68 \pm 1\ 93 \times 10^7$	$26\ 9 \pm 9\ 36$

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 O_2-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 ^3H_2O/ml, 0 5 µCi ^{14}C dextran/ml and 0 5 µCi ^{36}Cl/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 [ATP] + 0 5 [ADP]/[ATP] + [ADP] + [AMP] Kits are available commercially, which allow the determination of total ATP concentration using the luciferin-luciferase system ^{31}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, Covi-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 showe 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 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 β-hydroxy- β-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 ^{14}C HMG CoA for 30 mins at 37°C before termination of the reaction through the addition of hydrochloric acid and the addition of ^{3}H 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, Covi-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, Covi-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 O_2 An electrolytic cell in which oxygen is reduced to O_2 at an electrode can also be used However the indirect methods, which follow the rate of inhibition of the reaction between O_2 and a detector molecule are the most frequently used (Halliwell and Gutteridge, 1989) The usual O_2^- 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 benzyloxyphenoxazones), 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, cholestanetriol, Covi-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 3 4 1(b)) Cholestanetriol was found to have no effect on membrane integrity (Fig 3 4 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 Covi-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-ketocholelesterol 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(a)) 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 cholestanetriol 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 cholestanetriol 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 3b(b), 3 4 2b(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 cholestanetriol 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 concentration 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 cholestanetriol 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 Covi-ox led to a decrease in lipid peroxidation which, when examined in conjunction with the ability of Covi-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 cholestanetriol 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 Covi-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 cholestanetriol, although reported to be highly cytotoxic (Mahfouz et al, 1995, Verhagen et al, 1996, Ohtani et al, 1996, Gelissen et al, 1996), were found to have no effect on viability (cholestanetriol) or to effect viability only at higher concentrations (1200 and 2000 ppm 7-ketocholesterol)   Covi-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 cholestanetriol 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 coincubation 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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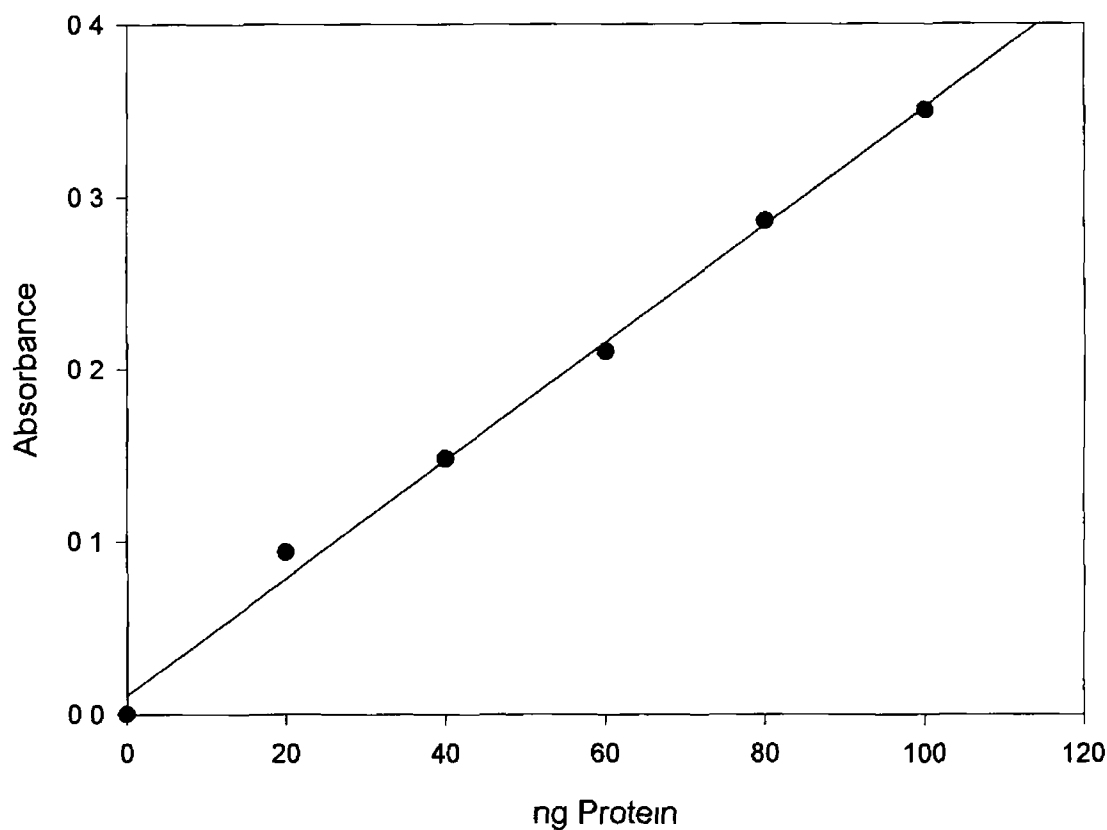
APPENDIX

A 1 LDH Assay
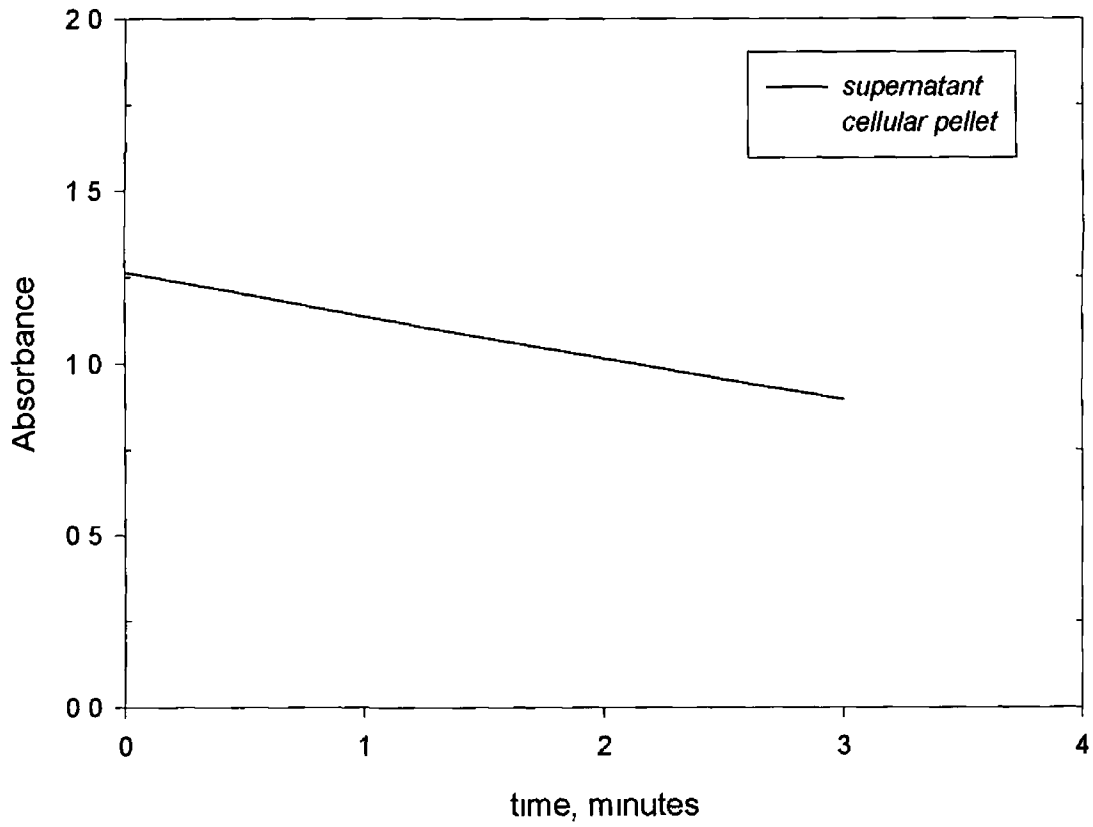
A 1 showing a typical graph recorded during the determination of LDH
activity in isolated hepatocytes The assay used is described in chapter 2
Supernatant indicates LDH activity in the cell medium which has leaked
from damaged cells Cellular pellet indicates LDH activity in intact hepatocytes

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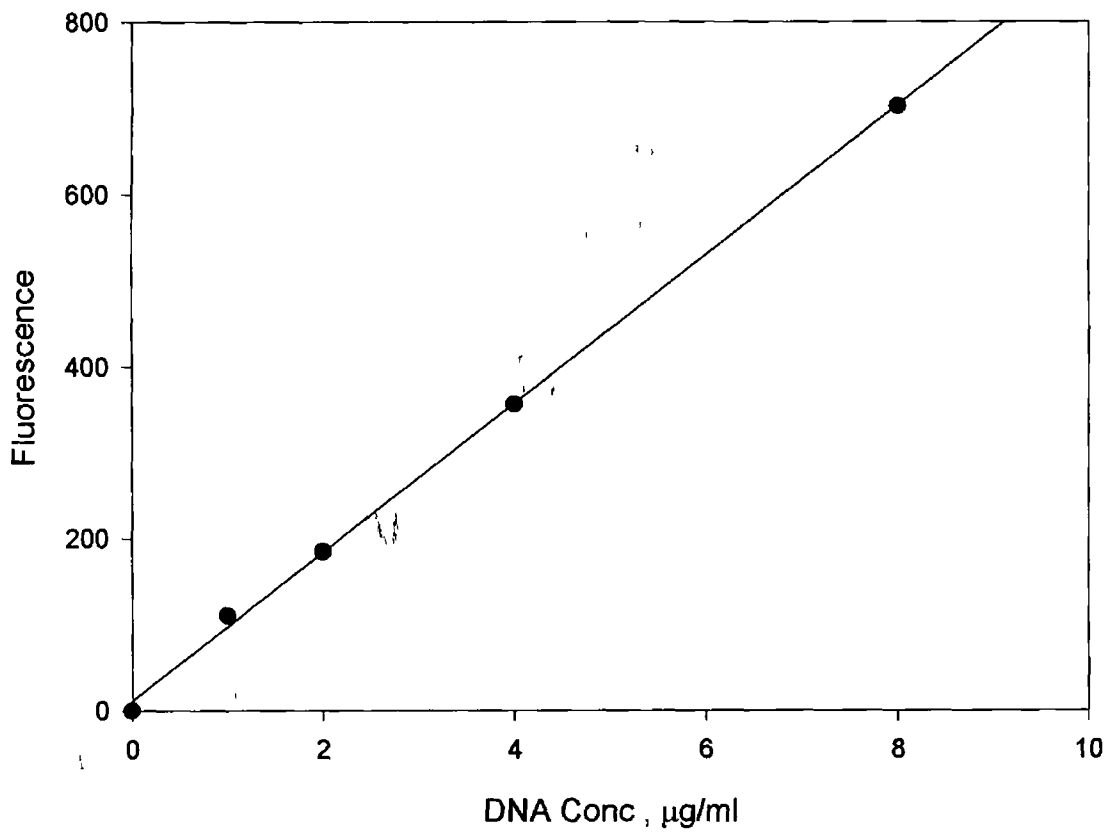
A 3 showing a typical standard curve used in the determination of the DNA content of isolated hepatocytes The DNA assay used is described in chapter 2
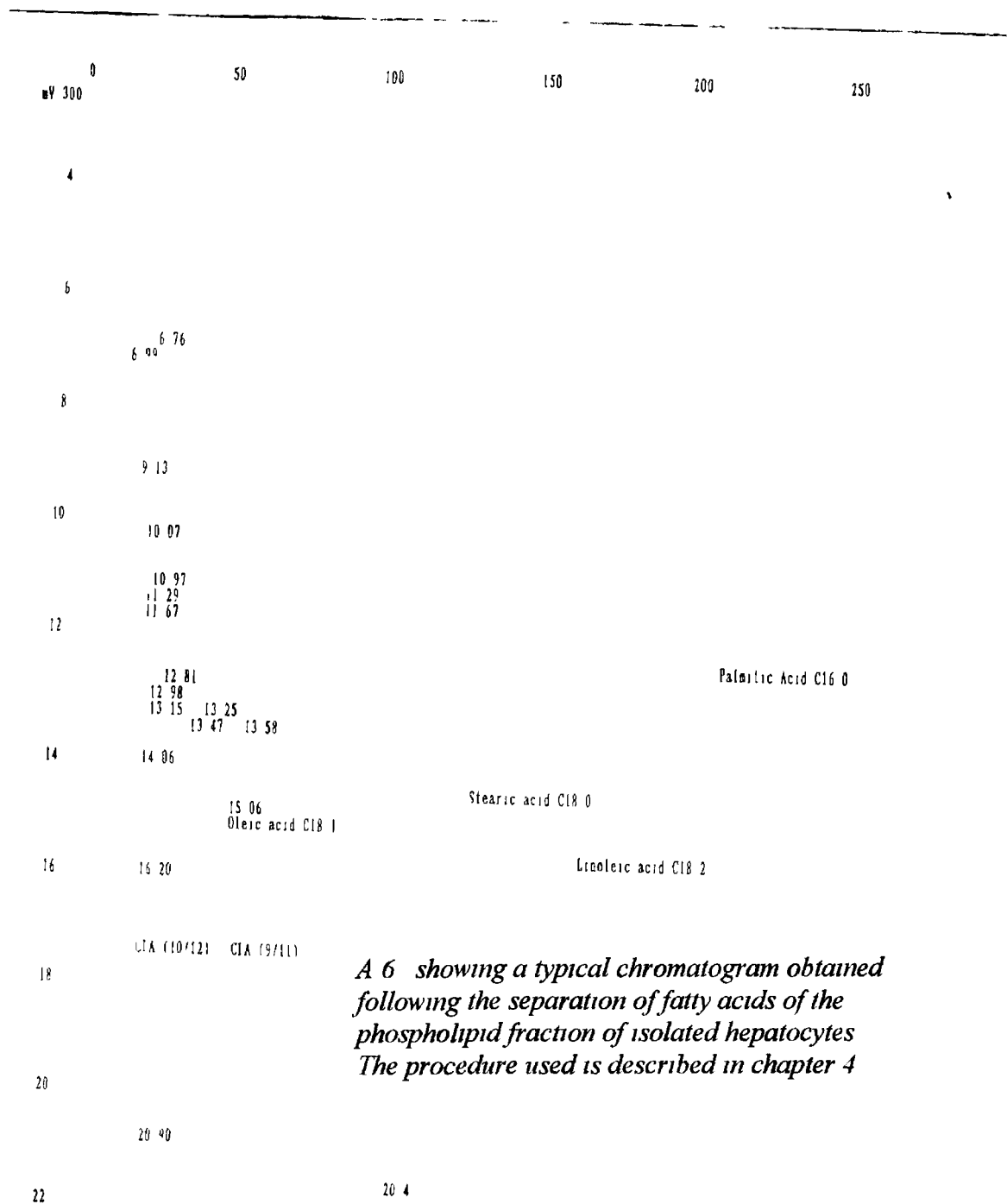
A 4 Standard Curve for ATP Assay
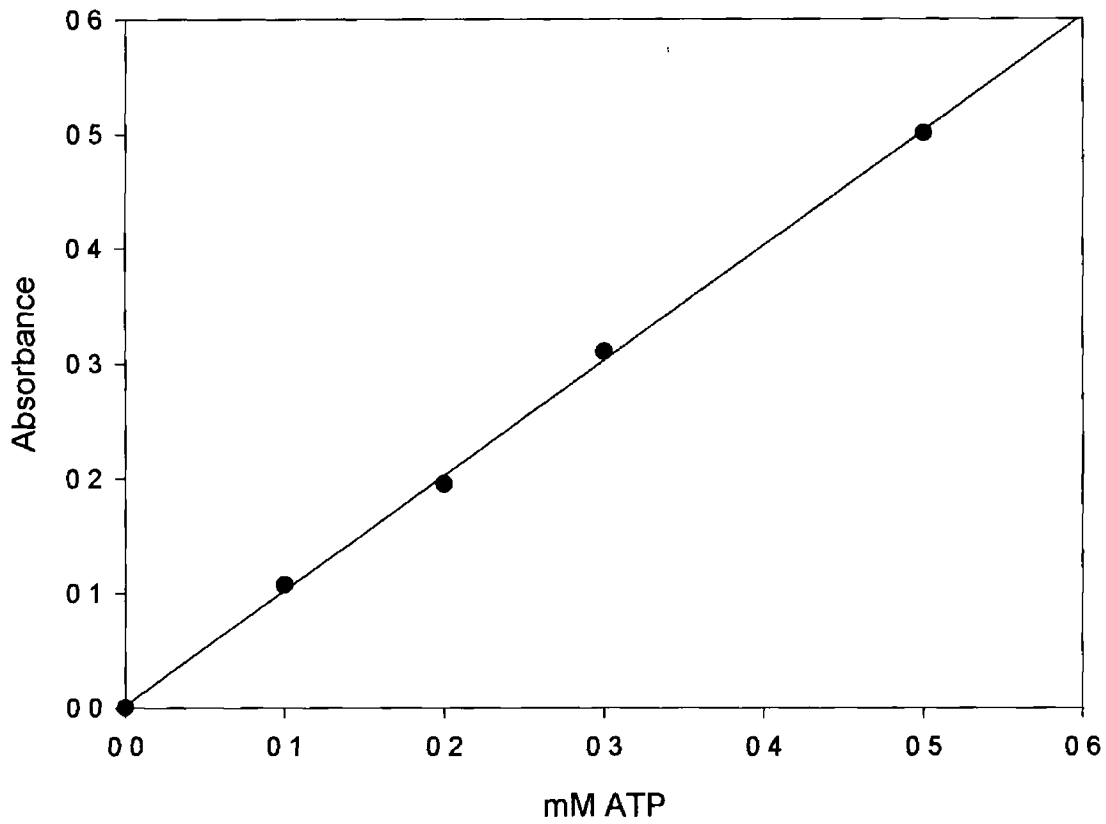
A 4 Showing a typical standard curve used in the assay to determine the ATP content of isolated hepatocytes The ATP assay is described in chapter 2

A 5 Standard Curve for SOD Assay
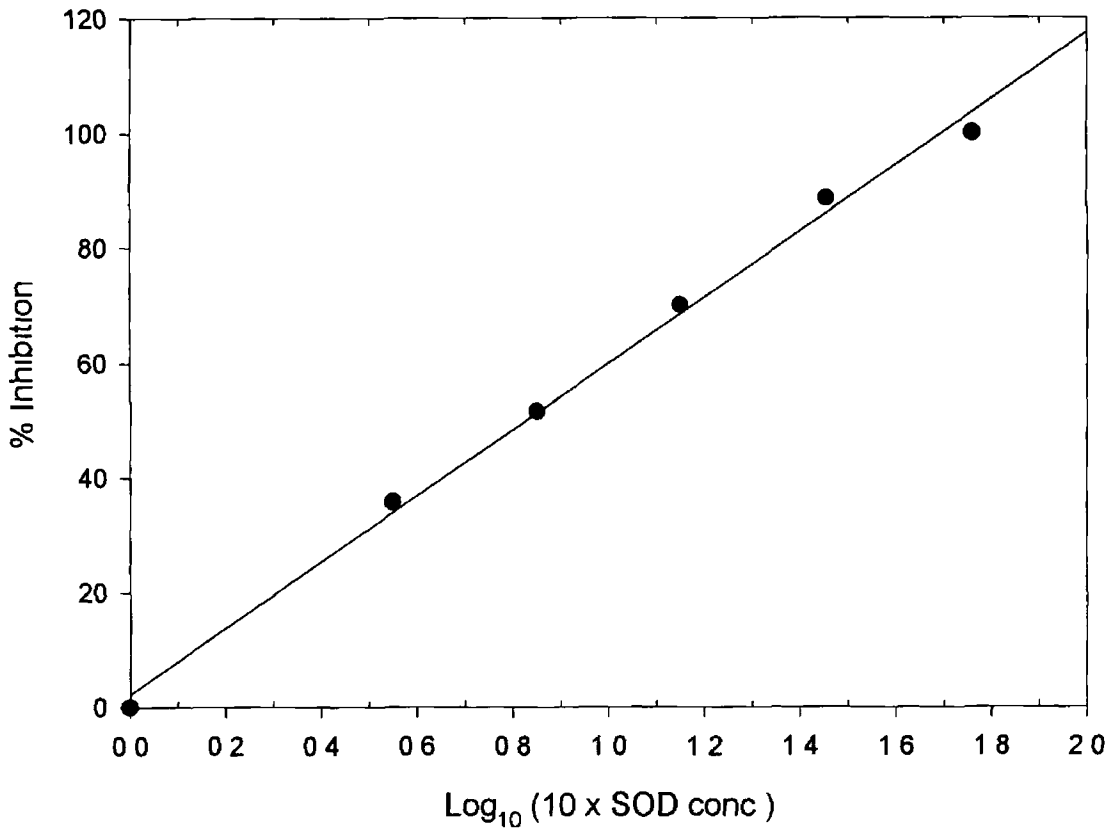
A 5 showing a typical standard curve used in the assay to determine the
SOD activity in isolated hepatocytes following a three hour incubation at
37degrees C and 5% CO_2 The assay is described in chapter 2

0   50   100   150   200   250
mV 300

4

6

6 76
6 qq

8

9 13

10

10 07

10 97
11 29
11 67

12

12 81
12 98
13 15   13 25
13 47   13 58

14   14 86

15 06   Stearic acid C18 0
Oleic acid C18 1

16   16 20   Linoleic acid C18 2

LIA (10/12)   CIA (9/11)

18

20

20 40

22   20 4

Palmitic Acid C16 0

*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*

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