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Free Radical Activity, Lipid Peroxidation and Antioxidant Status in Diabetes Mellitus

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# Free Radical Activity, Lipid Peroxidation and Antioxidant Status in Diabetes Mellitus

by

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Abstract

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The role of free radicals and antioxidants in human disease, particularly cardiovascular disease is an area of intensive research. Diabetes mellitus is the most common condition associated with increased oxidative stress and accelerated atherosclerosis. Increased levels of lipid peroxides and diminished antioxidant vitamin status have been reported in diabetic patients and are also implicated in the chronic complications of diabetes.

The autoxidation and glycoxidation reactions of glucose are sources of free radicals *in vitro* and a preliminary investigation that these reactions may be a source of free radicals *in vivo* was undertaken in patients admitted to hospital with severe hyperglycaemia or diabetic keto-acidosis. Plasma lipid peroxides were elevated 2-7 fold above the reference range, but decreased during the recovery period in these patients. Plasma urate and ascorbate levels decreased rapidly, whilst interestingly, α-tocopherol levels / lipid ratios were preserved. The study indicated the resilient nature of the antioxidant defences in plasma, although further studies are required in order to elucidate fully the role of autoxidation and glycoxidation reactions *in vivo*.

Insulin resistance and hyperinsulinaemia are also tightly linked with atherogenesis in type II diabetes and weight loss in obese subjects plays an important part in the reversal of insulin resistance. The safety and efficacy of two weight loss interventions — very low calorie diet (VLCD) and intensive conventional dietetic (ICD) therapy — on cardiovascular risk factors and indices of oxidative stress were investigated in obese diabetic and non-diabetic subjects.

The ICD therapy produced modest weight loss in patients with established diabetes with transient improvements in diastolic blood pressure and plasma ascorbate, but with a reduction in vitamin E / serum lipid ratios. The VLCD produced large and rapid weight loss in diabetic and non-diabetic patients with improvements in cardiovascular risk factors, lipid peroxides and vitamin E / serum lipid ratios, which were maintained after 12 months. Plasma ascorbate concentrations were significantly lower in diabetic patients than non-diabetic patients on the VLCD, indicating that formulated diets may require higher concentrations of vitamin C for diabetic patients and this requires further investigation. The VLCD successfully reversed type II diabetes and normalized plasma lipid peroxide levels in two newly diagnosed patients.

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List of Abbreviations

AA	Ascorbic acid
AGE(s)	Advanced glycosylation end product(s)
AH	Antioxidant
AOA	Antioxidant activity
A.R.	AnalaR® (reagent grade)
ATBC	The Alpha Tocopherol Beta Carotene Cancer Prevention Study
ATP	Adenosine triphosphate
AUFS	Absorbance units at full scale deflection
BB	Biobreeding rat
BDH	The British Drug Houses Limited
BHT	Butylated hydroxytoluene
BMI	Body mass index
CARET	Beta Carotene and Retinol Efficacy Trial
CCl_4 / $CCl_3\cdot$	Carbon tetrachloride / Carbon tetrachloride radical
C-H	Carbon-hydrogen bond
CH_2	Methylene group
CHD	Coronary heart disease
CML	Carboxymethyllysine
Cu^{2+} / Cu^+	Copper ions
Cu,ZnSOD	Copper-zinc superoxide dismutase
DCCT	Diabetes Control and Complications Trial
DHAA	Dehydroasorbic acid
DHBA	3,4-Dihydroxybenzylamine hydrobromide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
e / $\cdot$	Electron / Unpaired electron
EDRF	Endothelium-derived relaxing factor
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
et al.	*et alii*, and others
Fe^{2+} / Fe^{3+}	Ferrous ions / Ferric ions
FFA	Free fatty acids
FPG	Fasting plasma glucose
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
GPR	General purpose reagent
GSH	Glutathione (reduced form)

GSH-Px	Glutathione peroxidase
GSSG	Glutathione disulphide (oxidized form of GSH)
GST	Glutathione-S-transferases
H	Hydrogen atom
H+	Hydrogen ion
HDL	High-density lipoprotein
HMSO	Her Majesty's Stationary Office
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
ICD	Intensive conventional dietetic therapy
IGT	Impaired glucose tolerance
IU	International Units
LDL	Low-density lipoprotein
MDA	Malondialdehyde
MDA-TBA	Malondialdehyde-thiobarbituric acid adduct
min	Minutes
M^n / $M^{(n-1)+}$	Metal ions
Mn,SOD	Maganese containing superoxide dismutase
MPA	Metaphosphoric acid
NAD+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADP+	Nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
MOS	Dimethyl octyl silane
NO	Nitric oxide
NO_2	Nitrogen dioxide
NO_3^-	Nitrate
NOD	Non-obese diabetic mouse
NTFPO	National Task Force on the Prevention and Treatment of Obesity
O_2	Oxygen
$O_2^{\cdot-}$	Superoxide anion free radical
$^1O_2$	Singlet oxygen
ODS	Octadecyl silane
·OH	Hydroxyl radical
OH-	Hydroxyl ion
ONOO·	Peroxynitrite
ONOOH	Peroxynitrous acid
PAI-1	Plasminogen activator inhibitor type 1

PHGSH-Px	Phospholipid hydroperoxide glutathione peroxidase
PUFA	Polyunsaturated fatty acid
Q·	Semiquinone radical
R·	Lipid carbon-centred radical
RBP	Retinol binding protein
RH	Polyunsaturated fatty acid
RO·	Lipid alkoxyl radical
ROH	Alcohol
ROO·	Lipid peroxyl radical
ROOH	Lipid hydroperoxide
ROS	Reactive oxygen species
rpm	Revolutions per minute
RRR	All-racemic
RS·	Thiyl radical
RSO$_2$·	Sulphonyl radical
SD	Standard deviation
Se	Selenium
SOD	Superoxide dismutase
SNS	Sympathetic nervous system
STZ	Streptozotocin
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TEP	1,1,3,3-Tetraethoxypropane
α-TOC / α-TOC·	α-Tocopherol / α-Tocopheroxyl radical
TRAP	Total peroxyl-radical trapping antioxidant parameter
UK	United Kingdom
UKPDS	United Kingdom Prospective Diabetes Study
USA	United States of America
UV	Ultraviolet
UV/VIS	Ultraviolet / Visible
VLCD	Very low calorie diet
VLDL	Very-low density lipoprotein
v/v	Volume / volume
WHO	World Health Organization
w/v	Weight / volume
X·	Free radical
↑/↓	Increase / Decrease

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Author's Declaration

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Signed *Irene Balka*

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Chapter 1

Introduction

1. Introduction

1.1 Free Radicals

1.1.1 Historical overview

The term 'radical' was introduced by Lavoisier in 1789 to designate groups of elements which combined with oxygen in acids (Lavoisier 1789 cited by Ihde 1966). The term persisted and was used by chemists in the early nineteenth century to signify a group of atoms which remained unaltered through a series of reactions. Many attempts were made to isolate such compounds, particularly those containing trivalent carbon. In the 1860s, the vapour density method for the determination of molecular weights led to a reassessment of chemical structures thought to be radicals. Valency theory and the acceptance of the quadravalency of the carbon atom resulted in the abandonment of radical theories and their abnormal valency requirements (Ihde 1966). Thus, Gomberg's discovery in 1900 of the triphenylmethyl free radical, the first authenticated free radical, was treated with disbelief (Gomberg 1900). In the decades that followed, it was realized that simpler trivalent carbon atoms could have a fleeting existence and the presence of free radicals in the gaseous phase or in solution was unequivocally established in the period 1900-1930 (Ihde 1966).

In the 1940s, chemists at The British Rubber Producers' Research Association established the nature of the free radical reactions responsible for the rancidification of fats and oils (Bateman 1954). By the end of the 1960s, free radical technology was the basis of the polymer and plastic industry. In biological terms, an interest in free radicals only really began in 1968 with the discovery of superoxide dismutase (SOD), an enzyme in aerobic cells, whose specific role was the removal of the superoxide anion free radical (McCord and Fridovich 1969). The toxic effects of oxygen were well known and this discovery developed into the superoxide theory of oxygen toxicity, which stated that the toxic effects of oxygen were mediated by the superoxide free radical and that SODs provided an important defence against it (Fridovich 1975; Fridovich 1978). Prior to this discovery, Gerschman *et al*. (1954) had hypothesized that the damaging effects of oxygen and X-rays were attributed to the formation of oxidizing free radicals and Harman (1956) had proposed that the ageing process was caused by free radical reactions.

Today, a free radical is accepted as being either an atom or a molecule, possessing one or more unpaired electrons, that is capable of independent existence, the unpaired electron being one that is alone in an orbital (Halliwell and Gutteridge 1989). Free radicals are formed by the homolytic cleavage of a covalent bond (reaction 1.1), in contrast to heterolytic fission which produces ions (reaction 1.2). The free radical is represented by a dot ($\cdot$) that signifies the unpaired electron. Radicals may also be formed by the transfer of electrons from an electron rich donor to an electron acceptor.

$$A : B \rightarrow A \cdot + B \cdot \qquad\qquad (1.1)$$

$$A : B \rightarrow A : ^- + B + \qquad \text{or} \quad A + + B : ^- \qquad (1.2)$$

Radicals may be electrically charged or neutral. Most organic radicals are neutral, but all possess addition properties in order to achieve a more stable paired-electron status and are, as such, reactive species with short half-lives. The presence of unpaired electrons results in a small, permanent, magnetic moment and paramagnetic properties, enabling the direct detection of free radicals by electron spin resonance (ESR). Although ESR techniques are sensitive, the radicals under investigation are usually short-lived and detection is difficult. Such difficulties are overcome by the use of 'spin-traps', compounds which are non-radicals and have no ESR signal, but which are reactive towards other radicals forming 'spin-adducts', which are more persistent and detectable. Typical traps are the nitrones and nitroso compounds which form nitroxide spin-adducts (Janzen 1984; Rice-Evans *et al.* 1991). Unfortunately, ESR is not directly applicable to the study of free radicals in the clinical setting.

1.1.2 Biological implications

Molecular oxygen (O_2), as well as being essential to aerobic life, also imposes toxicity (Fridovich 1975; Cadenas 1989). The one electron reduction of O_2 from numerous biological sources, generates the superoxide anion free radical ($O_2 \cdot ^-$). This is a primary source of reactive radicals and damaging intermediates, which can result in the oxidation of proteins, lipids and deoxyribonucleic acid (DNA), thereby threatening cell integrity.

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Table 1.1 *Reactive oxygen species of biological importance (modified from Pryor 1994).*

Radical	Name	Substrate [a]	Half-life at 37°C (seconds)
$\cdot OH$	Hydroxyl radical	RH [b]	10^{-9}
$RO\cdot$	Lipid alkoxyl radical	RH	10^{-6}
$ROO\cdot$	Lipid peroxyl radical	RH	7
$R\cdot$	Lipid carbon centred radical	O_2	10^{-8}
H_2O_2	Hydrogen peroxide	- [c]	minutes
$O_2\cdot^-$	Superoxide anion radical	- [c]	10^{-5}
$^1O_2$	Singlet oxygen	H_2O	10^{-6}
$Q\cdot$ [d]	Semiquinone radical	O_2	Days
NO	Nitric oxide	- [e]	1-10
ONOO-	Peroxynitrite	-[f]	0.05-15
HOCl	Hypochlorous acid	- [g]	-

[a] Substrate chosen as a typical representative of a target molecule of the free radical.

[b] RH is a polyunsaturated fatty acid.

[c] The reactions of H_2O_2 and $O_2\cdot^-$ are limited by their reactions with enzymes.

[d] $Q\cdot$ represents a semiquinone radical as found in cigarette tar.

[e] Nitric oxide has several biological targets, e.g., haem proteins and $O_2\cdot^-$ (Bredt and Snyder 1994).

[f] Peroxynitrite is a potent oxidant, mediating numerous reactions (Beckman *et al.* 1994).

[g] Hypochlorous acid is a powerful oxidant produced by phagocytic cells with potent bactericidal activity.

The damage caused by reactive radicals to cellular components accumulates with age, has been postulated as being a major cause of ageing (Harman 1993; Kristal and Yu 1992) and is implicated in numerous degenerative diseases, including coronary heart disease (CHD), cancer, cataracts, inflammatory diseases, reperfusion injury and diabetes mellitus (Halliwell and Gutteridge 1990a; Kehrer 1993).

A group of related terms have been used in the scientific literature referring to free radicals of biological interest. These include oxygen radicals, oxygen derived radicals, oxygen free radicals, oxyradicals and the collective terms, reactive oxygen species (ROS) and reactive nitrogen species (Halliwell 1996). These terms include singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), which are not themselves free radicals, but are oxidizing agents and participate in cellular free radical reactions. The main ROS of biological interest are shown in Table 1.1.

1.1.3 Endogenous sources

Free radicals in living organisms originate from both endogenous and exogenous sources. A number of cellular sources of $O_2^{\cdot-}$ and other ROS have been identified:

1. The mitochondrial electron transport chains are a major source for the production of $O_2^{\cdot-}$ and H_2O_2 (Loschen *et al.* 1971; Boveris and Cadenas 1975). Under normal circumstances, the stepwise four electron reduction of O_2 results in the production of water, coupled with the formation of adenosine triphosphate (ATP). However, the occasional 'leakage' of electrons directly on to O_2 results in the production of $O_2^{\cdot-}$ (reaction 1.3). The formation of this radical should therefore be regarded as a normal by-product of aerobic respiration (Fridovich 1989).

$$O_2 + e \rightarrow O_2^{\cdot-} \tag{1.3}$$

2. The electron transport chains of the endoplasmic reticulum are also capable of producing $O_2^{\cdot-}$. Cytochrome P450, a terminal component of electron transport chains found in liver endoplasmic reticulum (microsomes), is important for the detoxification of xenobiotic compounds and hydroxylation reactions involved in synthesis. These reactions require the activation of O_2 by the transfer of electrons from nicotinamide adenine dinucleotide phosphate (NADPH); the occasional leakage of electrons results in the formation of $O_2^{\cdot-}$ (Bast 1986; White 1991). Free radical intermediates are also produced during the detoxification of xenobiotics, the main sources being plant phenolics of dietary origin, drugs and halogenated compounds found in pesticides and environmental pollutants (Stohs 1995). A massive load of xenobiotics, e.g., during carbon tetrachloride (CCl_4) poisoning, results in the production of the $CCl_3^{\cdot}$ radical, which rapidly promotes lipid peroxidation in cellular membranes, causing liver damage (Slater 1982; Comporti 1993).

3. Phagocytic cells are specialized in the production of $O_2^{\cdot-}$ and other ROS. Activation of these cells produces a sudden rise in O_2 consumption (the respiratory burst) and $O_2^{\cdot-}$ is produced by the one electron reduction of O_2, catalysed by a plasma-membrane-bound, NADPH-dependent oxidase (Babior *et al.* 1973; Babior 1987). The $O_2^{\cdot-}$ produced dismutes

to H_2O_2, which is utilized by myeloperoxidase to oxidize chloride to HOCl (Weiss 1989). Thus, phagocytes produce a battery of oxidants, including $O_2^{\cdot-}$, H_2O_2, HOCl and also the hydroxyl radical ($\cdot$OH) and nitric oxide (NO) (Marletta *et al*. 1988; Hurst and Barrette 1989; Ramos *et al*. 1992). The importance of these ROS is accentuated by the genetic disorder chronic granulomatous disease, where the inability of the NADPH-oxidase to produce $O_2^{\cdot-}$ results in impaired bactericidal action and persistent infections (Babior 1987). Conversely, tissue damage results if such reactive species are not tightly controlled. Consequently, chronic infections which result in inflammation and several disease processes, including atherosclerosis and type I diabetes are associated with excessive phagocytic activity (Weiss 1989; Kröncke *et al*. 1991; Kehrer and Smith 1994; Bottazzo *et al*. 1985; Foulis *et al*. 1986).

4. Certain enzymes are also capable of producing free radical intermediates. For example: the synthesis of prostaglandins, leukotrienes and other eicosanoids from arachidonic acid, catalysed by cyclo-oxygenase and lipoxygenase enzymes, is a controlled form of lipid peroxidation during which peroxyl radical intermediates are formed (Gurr and Harwood 1991; White 1991); peroxisomes contain the enzymes urate oxidase and D-amino acid oxidase which produce H_2O_2 as a by-product (van den Bosch *et al*. 1992); the enzyme xanthine oxidase, present in many tissues, oxidizes hypoxanthine to xanthine then urate with the formation of $O_2^{\cdot-}$ and H_2O_2, a reaction widely used for the generation of $O_2^{\cdot-}$ *in vitro* (Rice-Evans *et al*. 1991). Levels of xanthine oxidase are normally low in human tissues (since the enzyme exists as the dehydrogenase which uses NAD+, not O_2, as the electron acceptor), but can increase during periods of tissue ischaemia, with the potential of mediating free radical tissue injury, upon the introduction of O_2 during reperfusion (McCord 1985; Granger 1988; Omar *et al*. 1991; Bulkley 1994).

5. Oxygen is also activated by the absorption of energy, forming $^1O_2$ (Khan 1976). Excitation can be achieved when pigments absorb light and transfer energy to O_2 (photoexcitation), or by chemiexcitation during enzymatic reactions and radical interactions (Murphy and Sies 1990). For example, $^1O_2$ can be formed during the disproportionation of lipid peroxyl radicals (section 1.3.3) or during the metabolism of hydroperoxides (Naqui *et*

al. 1986). Although $^1O_2$ is not a free radical, its reactivity with other biological molecules is increased so that it is capable of damaging proteins, lipids and DNA (Foote *et al.* 1984). Singlet oxygen has been implicated in skin photosensitivity, lung oxidant injury and damage to the retina by over exposure to bright light (Halliwell and Gutteridge 1989).

6. Nitric oxide is another endogenously produced free radical, formed during the oxidation of L-arginine to L-citrulline by nitric-oxide synthase enzymes, which serves as an important physiological messenger molecule (Noack and Murphy 1991; Prince and Gunson 1993; Masters 1994; Bredt and Snyder 1994). In the 1980s, several areas of research came together revealing the involvement of NO in biological systems. It was first recognized that the vascular endothelium synthesized NO, a factor previously known as the endothelium-derived relaxing factor (EDRF), which mediated the relaxation of smooth muscle cells and prevented the aggregation and adhesion of platelets to the endothelium (Furchgott and Zawadzki 1980; Palmer *et al.* 1987; Ignarro *et al.* 1987; Palmer *et al.* 1988). It is now accepted that NO is produced by a variety of cells, including neuronal, macrophages, smooth muscle, platelets and fibroblasts and mediates diverse biological functions, such as the regulation of vascular tone and blood pressure, neurotransmission and the bactericidal and tumoricidal actions of macrophages (Marletta *et al.* 1988; Marletta 1989; McCall *et al.* 1989; Snyder and Bredt 1992; Moncada and Higgs 1993).

Besides mediating normal functions, fluctuations in the production of NO have been implicated in the pathogenesis of hypertension, septic shock, inflammation and atherosclerosis (Moncada and Higgs 1993; Anggard 1994; White *et al.* 1994). The high reactivity of NO with $O_2 \cdot^-$ and other radicals (Huie and Padmaja 1993; Darley-Usmar *et al.* 1995) are reactions which may have important implications in atherosclerosis, since NO appears to possess both antioxidant and pro-oxidant properties (Rubbo *et al.* 1994). The rapid reaction of NO with lipid peroxyl radicals resulted in the formation of stable products and demonstrated an inhibitory effect of NO upon lipid peroxidation (Rubbo *et al.* 1994). Similarly, NO was found to exert a protective role towards low-density lipoproteins (LDL) against oxidative modification (Jessup *et al.* 1992; Yates *et al.* 1992; Hogg *et al.* 1993a). However, where both NO and $O_2 \cdot^-$ are formed, e.g., by endothelial cells and macrophages,

NO can exert a pro-oxidant effect. The combination of NO and $O_2^{\cdot-}$, produces peroxynitrite $(ONOO^-)$ (Blough and Zafiriou 1985) and its conjugate acid, peroxynitrous acid (reactions 1.4 and 1.5), potent oxidants which mediate many reactions (Beckman *et al.* 1990; Beckman *et al.* 1994).

$$NO \quad + \quad O_2^{\cdot-} \quad \rightarrow \quad ONOO^- \qquad\qquad (1.4)$$

$$ONOO^- + \quad H^+ \quad \leftrightarrow \quad ONOOH \qquad\qquad (1.5)$$

$$ONOOH \quad \rightarrow \quad \cdot OH \quad + \quad NO_2 \quad \rightarrow \quad NO_3^- \quad + \quad H^+ \qquad (1.6)$$
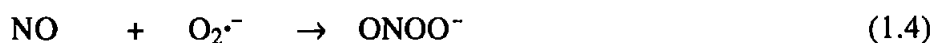
The cytotoxic potential of $ONOO^-$ was highlighted by Beckman *et al.* (1990) who demonstrated that the decomposition of $ONOO^-$ could produce oxidants with reactivity comparable to that of $\cdot OH$ (reaction 1.6). In fact, $ONOO^-$ has been shown to initiate lipid peroxidation in liposomes (Radi *et al.* 1991a; Rubbo *et al.* 1994) and in LDL (Darley-Usmar *et al.* 1992; Hogg *et al.* 1993b). Thus, it has been suggested that the formation of $ONOO^-$ may exacerbate atherosclerosis (White *et al.* 1994). Apart from potentially initiating lipid peroxidation and the oxidative modification of LDL, $ONOO^-$ can deplete plasma anti-oxidants, including vitamins E and C (de Groot *et al.* 1993; Hogg *et al.* 1993b; Van der Vliet *et al.* 1994), oxidize proteins (Radi *et al.* 1991b) and bring about the release of copper ions from caeruloplasmin, which may then initiate the oxidation of LDL (Swain *et al.* 1994).

7. Several transition metal ions qualify as free radicals and are of importance *in vivo* because of their ability to catalyse reactions (Halliwell and Gutteridge 1984). The dismutation of $O_2^{\cdot-}$, catalysed by SOD, results in the formation of H_2O_2 (reaction 1.7).

$$2O_2^{\cdot-} \quad + \quad 2H^+ \quad\quad \rightarrow \quad H_2O_2 \quad + \quad O_2 \qquad\qquad (1.7)$$

Hydrogen peroxide is not a free radical, but is capable of giving rise to the highly reactive $\cdot OH$, especially under the influence of transition metal ions. Hydroxyl radicals can be generated through a $O_2^{\cdot-}$ driven Fenton reaction, where $O_2^{\cdot-}$ reduces ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) via an intermediate perferryl complex, $Fe^{3+} - O_2^{\cdot-} \leftrightarrow Fe^{2+} - O_2$, (reaction 1.8) (Halliwell and Gutteridge 1990a). The ferrous ions then act as electron donors, rapidly reducing H_2O_2 by the Fenton reaction to the hydroxyl ion (OH^-) and $\cdot OH$, reaction 1.9 (Koppenol 1993; Goldstein *et al.* 1993).

$$O_2^{\bullet-} \quad + \quad Fe^{3+} \quad \rightarrow \quad Fe^{2+} \quad + \quad O_2 \qquad\qquad (1.8)$$

$$H_2O_2 \quad + \quad Fe^{2+} \quad \rightarrow \quad Fe^{3+} \quad + \quad OH^- \quad + \quad {\bullet}OH \qquad (1.9)$$
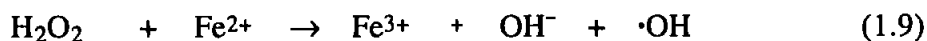
Consequently, under physiological conditions the interaction of $O_2^{\bullet-}$ and H_2O_2, in the presence of catalytic metal ions, may result in the formation of ${\bullet}OH$. The net reaction (1.10) is often called the Haber-Weiss reaction (Haber and Weiss 1934; Walling 1975).

$$\overset{Fe^{2+}/Cu^+}{2O_2^{\bullet-} \quad + \quad H_2O_2 \quad \rightarrow \quad O_2 \quad + \quad OH^- \quad + \quad {\bullet}OH} \qquad (1.10)$$
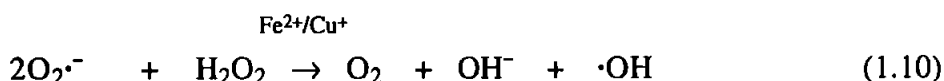
The formation of ${\bullet}OH$, *in vivo*, may be limited by the supply of catalytic metal ions and tissue injury by any mechanism can exacerbate free radical reactions, if metal ions are released (Gutteridge 1986; Halliwell and Gutteridge 1990a).

1.1.4   Exogenous sources

In addition to the cellular formation of ROS, exogenous sources may increase the free radical load. For example, ionizing radiation of the skin can produce free radicals, cigarette smoke contains many radicals and oxidizing species and the absorption from the diet of large quantities of iron or copper salts, or xenobiotics can increase the endogenous production of free radicals (Pryor and Stone 1993; Stohs 1995; Halliwell 1996).

In summary, it is through the formation of $O_2^{\bullet-}$, H_2O_2, $ONOO^-$ and ${\bullet}OH$ in particular, that the toxicity of oxygen is mediated (Halliwell 1996). Hydroxyl radicals are highly reactive and will attack any molecule in the immediate vicinity, protein, lipid, carbohydrate or nucleic acid and are capable of causing indiscriminate cellular damage (Slater 1984a; Slater *et al.* 1987; Stadtman 1993; Cadenas 1995). As a consequence of the potential toxic effects of oxygen, aerobic organisms have evolved numerous antioxidant defences to limit the formation and damage caused by free radicals. These defences act at different stages in free radical reactions, e.g., by removing key reactive species, binding metal ions, terminating free radical chain reactions and quenching excited molecules.

1.2 Antioxidant Defences

1.2.1 Cellular antioxidants

At the cellular level, the antioxidant defences operate by: (1) preventing the initial formation of radicals, (2) removing the intermediates of oxygen reduction, (3) intercepting radicals once they have been formed and (4) repairing or eliminating molecules damaged by free radical activity (Fridovich 1989).

1. The initial formation of $O_2^{\cdot-}$ is prevented by cytochrome oxidase, the terminal oxidase of the mitochondrial electron transport chain, which carries out the tetravalent reduction of O_2, without releasing reactive oxygen intermediates from its active site (Chance et al. 1979). Cytochrome oxidase has also been attributed with possessing SOD and peroxidase activity (Naqui et al. 1986).

The transition metal ions, especially iron and copper, are accepted as being involved in the formation of free radicals in vivo, by catalysing the decomposition of H_2O_2 and organic hydroperoxides (Halliwell and Gutteridge 1984). For this reason, metal ions are tightly sequestered by proteins, making them unavailable to participate in the generation of free radicals. Such proteins are also considered as preventative antioxidants (Frei et al. 1988). In extracellular fluids, these proteins form major antioxidant defences (Table 1.3, page 13). Intracellularly, iron is bound to ferritin or haemosiderin, or is chelated to various constituents (citrate or phosphate esters), to minimize the occurrence of free metal ions (Halliwell and Gutteridge 1984).

2. The removal of key reactive species, $O_2^{\cdot-}$, H_2O_2, is achieved by SOD and peroxidase enzymes (Table 1.2). The SODs are ubiquitous in aerobic organisms, an indication of how widespread the formation of $O_2^{\cdot-}$ may be, but differ in structure and metal ions present at their active sites. Eukaryotic cells possess a copper and zinc SOD (Cu,Zn SOD), located in the cytoplasm and a manganese containing SOD (Mn SOD), within the mitochondrial matrix (Fridovich 1995). The SODs provide an important defence against the toxicity of O_2, by catalysing the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2. (McCord and Fridovich 1969).

The H_2O_2 generated by the dismutation of $O_2 \cdot^-$ and as a by-product of enzymatic reactions is removed by catalase and various peroxidases (Chance *et al.* 1979). Catalase is specific for H_2O_2 and is predominantly located within single-membrane organelles, the peroxisomes, which contain H_2O_2 generating enzymes, such as urate oxidase, glucose oxidase and the flavoprotein dehydrogenases involved in the ß-oxidation of fatty acids (van den Bosch *et al.* 1992; Reddy and Mannaerts 1994). Catalase reduces H_2O_2 to H_2O and O_2, and shares this property with other peroxidases (Table 1.2).

The most important peroxidases are the glutathione peroxidases (GSH-Px), which require selenium (Se) as a cofactor and are dependent upon glutathione (γ-L-glutamyl-L-cysteinyl-glycine; GSH) for the supply of reducing equivalents (Stadtman 1991). Thus, GSH-Px catalyses the reduction of H_2O_2 at the expense of GSH, Table 1.2. In addition to H_2O_2, GSH-Px is also capable of reducing organic hydroperoxides, including fatty acid, nucleotide and steroid hydroperoxides to the corresponding alcohols (Chance *et al.* 1979). However, fatty acid hydroperoxides must be released from membranes by the action of phospholipase A_2 before GSH-Px can act; although, a second Se-containing enzyme, phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px), has been discovered in mammals which has the unique ability of directly reducing lipid hydroperoxides within membranes (Ursini *et al.* 1982; Ursini *et al.* 1985; Ursini *et al.* 1991).

Glutathione peroxidase appears to be more important than catalase for removing H_2O_2, because it is found within the same subcellular locations as SOD. Comparative studies have also shown GSH-Px to be more efficient than catalase and SOD under experimental conditions of oxidative stress. Any reduction in the activity of GSH-Px rendered cells more susceptible to oxidative damage (Toussaint *et al.* 1993). Glutathione peroxidases are, therefore, key enzymes, protecting cells against damage caused by the decomposition of H_2O_2 and lipid hydroperoxides.

Table 1.2 *The major cellular antioxidant enzymes.*

Enzyme	Reaction
Superoxide Dismutase (Cu, Zn-SOD; MnSOD)	$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$
Glutathione Peroxidase (GSH-Px)	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$ $ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$
Phospholipid Hydroperoxide Glutathione Peroxidase (PHGSH-Px)	$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$
Glutathione-S-Transferase (GST)	$ROOH + 2GSH \rightarrow ROH + H_2O + GSSG$

The glutathione-S-transferases (GST), a group of non-Se containing iso-enzymes, have numerous functions and are involved with the detoxification of xenobiotics, via conjugation with GSH. These enzymes also reduce organic hydroperoxides, including lipid hydroperoxides to the corresponding alcohols at the expense of GSH, Table 1.2 (Ahmad 1995). The GSH-Pxs and GST enzymes are dependent upon GSH for their activity; hence, the supply of GSH must be replenished. This is achieved by the action of an NADPH-dependent enzyme, glutathione reductase, which reduces the oxidized glutathione (GSSG) back to GSH, Figure 1.3 (page 18). This enzyme plays an indirect part in cellular antioxidant defences. Glutathione also reacts directly with $O_2^{\cdot-}$, $^1O_2$ and $\cdot OH$ and, therefore, acts as a water-soluble antioxidant, as well as a physiological reducing agent (Meister and Anderson 1983).

3. The antioxidant enzymes form an important line of defence by preventing the accumulation of $O_2^{\cdot-}$ and removing H_2O_2, thereby preventing the formation of $\cdot OH$ and other highly reactive radicals. However, if these reactants escape removal, leading to the

formation of other ROS, non-enzymatic, small molecular-weight antioxidants become important for the direct scavenging of these initiating / propagating radicals. The major water and lipid-soluble antioxidants are listed in Table 1.4 (page 14) and their interactions are shown in Figure 1.3 (page 18).

4. If ROS, such as ·OH are formed, damage to surrounding molecules is probably unavoidable. Thus, the final form of defence against free radicals comes from enzymes, which repair or eliminate damaged molecules. In healthy cells, damaged DNA bases are constantly removed and replaced by repair enzymes, the hydroxylated bases being excreted in the urine (Breimer 1991; Demple and Harrison 1994). Damaged proteins are degraded by proteases (Davies 1987; Marcillat *et al.* 1988) and oxidized lipids by the activity of phospholipases (Malis *et al.* 1990; van den Berg *et al.* 1993).

1.2.2 Plasma antioxidants

Plasma functions as a transporter of a wide variety of compounds, especially those of dietary origin and cellular metabolism. It is exposed to O_2 and oxidants derived from endogenous and exogenous sources. By scavenging reactive molecules, plasma serves to protect the endothelial lining of the vasculature and its own contents, especially the lipid transporting lipoprotein particles (Stocker and Frei 1991). Imbalances resulting in damage to the vasculature, or oxidation of the lipoprotein particles, are events of prime importance in the aetiology of atherosclerosis (Steinberg *et al.* 1989).

The antioxidant defences in plasma differ from the defences found within cells in that they lack the antioxidant enzymes SOD, GSH-Px and catalase (Halliwell and Gutteridge 1990b). However, a distinct tetrameric Cu,Zn SOD, a glycoprotein with heparin binding affinity, has been described which appears to be bound to the endothelial cell surfaces (Marklund 1982; Karlsson and Marklund 1987). A Se-dependent GSH-Px, different from the enzyme present within erythrocytes, has also been described in human plasma (Maddipati and Marnett 1987; Takahashi *et al.* 1987).

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Table 1.3 *Antioxidant proteins present in plasma (modified from Stocker and Frei 1991).*

Protein (plasma concentration)	Function
Albumin (30-60 g/l)	Albumin transports a wide range of substances, such as fatty acids and bilirubin and also has specific binding sites for Cu^{2+} ions. Albumin bound Cu^{2+} is still redox active and may participate in the generation of ·OH, which reacts with the protein itself, in a 'site-specific' manner (Halliwell 1988). The protein functions as a 'sacrificial antioxidant', thereby protecting other important targets and is rapidly replaced. Albumin also possesses sulphydryl groups which can react with free radicals (Wayner *et al.* 1987).
Transferrin (1.2-3.3 g/l)	Transferrin binds Fe^{3+} ions, for transport and delivery to cells, making iron unavailable to participate in redox reactions. The protein is only 20-30% saturated with iron, the excess binding capacity means that no free iron is found in plasma (Gutteridge *et al.* 1981a).
Ferritin	The iron-storage protein ferritin is found intracellularly and oxidizes Fe^{2+} to Fe^{3+} (Halliwell and Gutteridge 1984), and mediates transfer to transferrin. However, iron can be released from ferritin to promote redox reactions (Halliwell and Gutteridge 1986; Thomas *et al.* 1985).
Caeruloplasmin (0.18-0.4 g/l)	This copper-transporting protein, inhibits copper / iron stimulated lipid peroxidation and possesses ferroxidase activity, converting Fe^{2+} to Fe^{3+} (Gutteridge *et al.* 1980; Gutteridge 1983), which may be incorporated back to ferritin or transferrin (Halliwell & Gutteridge 1984). It also scavenges $O_2 \cdot^-$ (Goldstein *et al.* 1979a; Samokyszyn *et al.* 1989).
Lactoferrin (0.2 mg/l)	Lactoferrin is released from activated neutrophils and has a similar function to transferrin by binding Fe^{3+} ions and making them unavailable to participate in redox reactions (Gutteridge *et al.* 1981b; Baldwin *et al.* 1984; Winterbourn 1983; Lönnerdal and Iyer 1995).
Haptoglobin (0.5-3.6 g/l) Haemopexin (0.6-1.0 g/l)	Haptoglobins bind haemoglobin (Hb) and met-Hb, preventing these proteins from releasing iron and initiating lipid peroxidation. Similarly, haemopexins bind free haem and prevent the release of haem iron. However, protein damage by peroxyl radicals may release bound iron (Gutteridge 1987; Gutteridge and Smith 1988).

A major contribution to the extracellular antioxidant defences in plasma comes from the proteins that bind metal ions, preventing them from participating in redox reactions and generating free radicals (Table 1.3) (Dormandy 1980; Wayner *et al.* 1985; Wayner *et al.* 1987; Halliwell and Gutteridge 1990b). In addition to the proteins, the small molecular-weight antioxidant molecules listed in, Table 1.4, serve to protect the lipid and water-soluble components of plasma, by directly intercepting free radicals. The most important of these antioxidants, vitamins A, C and E are the main interest in this thesis.

Table 1.4 *Non-enzymatic, small molecular-weight antioxidants (from Sies and Stahl 1995).*

Antioxidant	Plasma concentration
Lipid-soluble	μmol/l
RRR-α-Tocopherol	15 - 40
δ-Tocopherol	3 - 5
all-*trans*-retinol	1.5 - 2.8
α-Carotene	0.05 - 0.1
ß-Carotene	0.3 - 0.6
Lycopene	0.5 - 1.0
Lutein	0.1 - 0.3
Zeaxanthin	0.1 - 0.2
Ubiquinol-10	0.4 - 1.0
Water-soluble	
Ascorbic acid	30 - 150
Glutathione	1 - 2
Uric acid	160 - 450
Bilirubin	5 - 20

(i)   Lipid-soluble antioxidants

Vitamin E

The term 'vitamin E' refers to a group of fat-soluble compounds (tocopherols and tocotrienols, Figure 1.1), with the biological activity of α-tocopherol (Diplock 1985). The most important biologically active form, RRR-α-tocopherol (see Appendix 1 for nomenclature and International Units (IU)), is an essential nutrient for all mammalian species, required to support reproduction in rodents and prevent deficiency symptoms in man, such as reduced erythrocyte lifespan (haemolysis), neuromuscular defects and abnormal platelet activity (Diplock 1985; Machlin 1991; Basu and Dickerson 1996).

The principal function of α-tocopherol is to maintain the integrity of cell membranes by preventing the peroxidation of the polyunsaturated fatty acids (PUFAs) (Diplock 1985). The tocopherol molecule performs this function as an antioxidant, by donating its phenolic H-atom to a lipid peroxyl radical (ROO·), thereby terminating the chain reaction of lipid peroxidation (reaction 1.11). The resulting tocopheroxyl radical is sufficiently stable to prevent the continuation of the free radical chain reaction and can be reduced back to

tocopherol by reducing agents. The tocopheroxyl radical can also undergo a second oxidation and react with a second ROO· (reaction 1.12), resulting in the formation of a tocopheryl quinone (Figure 1.1). Thus, one molecule of α-tocopherol is capable of scavenging two lipid peroxyl radicals (Burton and Ingold 1981; Liebler and Burr 1995). The tocopheryl quinone reacts reversibly to form a hydroquinone, which can be conjugated to yield glucuronic acid and the product excreted into bile (Basu and Dickerson 1996).

$$\text{α-TOH} + \text{ROO·} \rightarrow \text{α-TO·} + \text{ROOH} \qquad (1.11)$$

$$\text{α-TO·} + \text{ROO·} \rightarrow \text{Inactive products} \qquad (1.12)$$
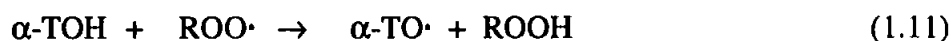
Burton and Ingold (1981) established that α-tocopherol was the most efficient biologically active form and the most important lipid-soluble antioxidant in plasma, erythrocytes and tissues (reviewed by Burton and Traber 1990; van Acker *et al.* 1993; Traber and Sies 1996). In addition to its role as the main lipid-soluble 'chain-breaking' antioxidant, α-tocopherol is also capable of scavenging $^1O_2$ (Kaiser *et al.* 1990; Fahrenholtz *et al.* 1974 and Foote *et al.* 1974, cited by Traber and Sies 1996) and NO (de Groot *et al.* 1993).

In plasma, almost all of the α-tocopherol is located within the lipoprotein particles and is strongly correlated with lipid content (Horwitt *et al.* 1972; Thurnham *et al.* 1986), with approximately one α-tocopherol molecule protecting 200 fatty acids in LDL (Esterbauer *et al.* 1991). The regeneration of α-tocopherol is, therefore, an important process and several mechanisms may operate to reduce the tocopheroxyl radical, although the precise mechanisms have not been established *in vivo*. The tocopherol molecule is anchored within membranes by the phytyl group, with the chroman group positioned towards the membrane surface and the aqueous environment. This positioning may enable the molecule to interact with aqueous antioxidants. Indeed, ascorbate and GSH were reported to interact with and regenerate the tocopheroxyl radical, thereby increasing its efficiency as an antioxidant (Packer *et al.* 1979; McCay 1985; Sato *et al.* 1990; Niki *et al.* 1991; Ingold *et al.* 1993; Buettner 1993). Similarly, lipid-soluble antioxidants, such as ubiquinol, may reduce the tocopheroxyl radical (Maguire *et al.* 1992), or interact in other ways which synergistically inhibit lipid peroxidation, as demonstrated by ß-carotene (Palozza and Krinsky 1992a).
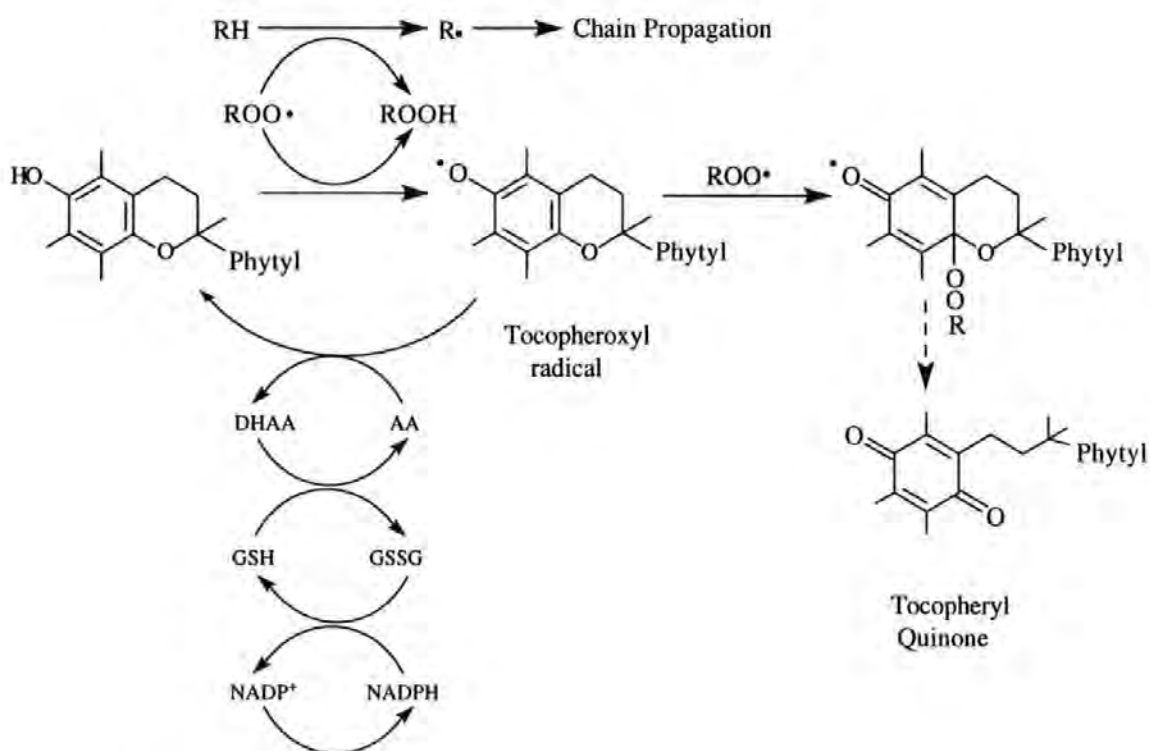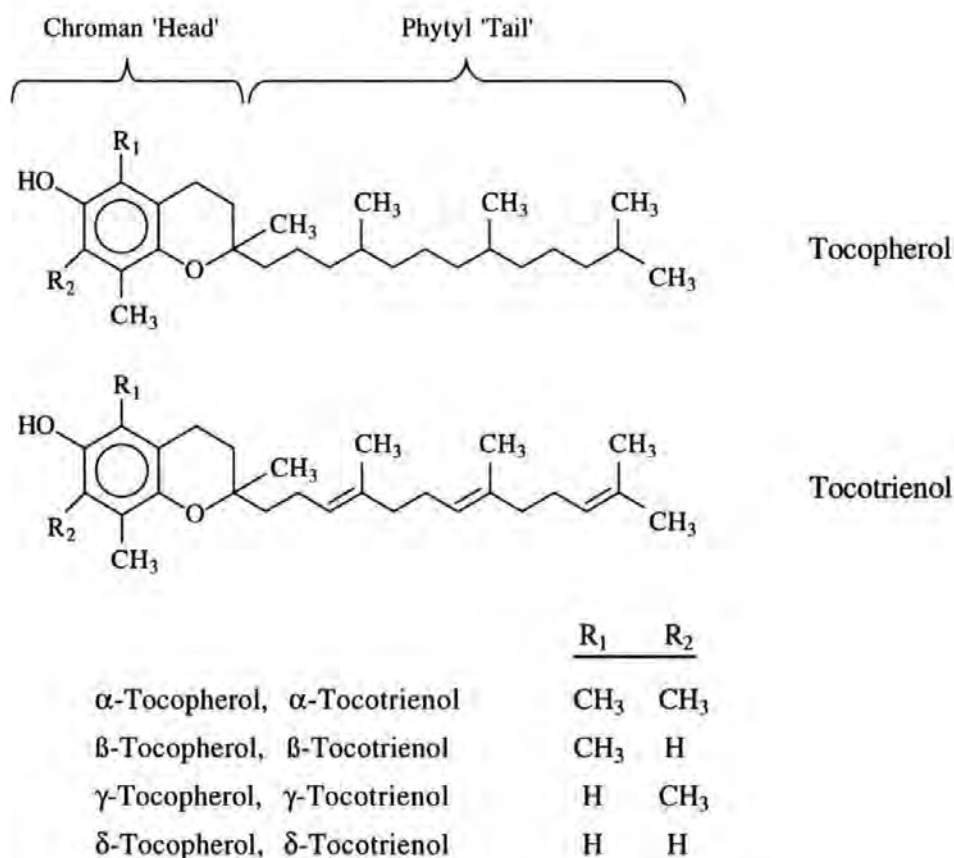
Figure 1.1 *Structures of tocopherols, tocotrienols and the antioxidant action of vitamin E (modified from Sies and Stahl 1995).*

Lipid Soluble Antioxidants

Retinol

Beta Carotene

Lycopene

Quercetin

Ubiquinol-10

Water Soluble Antioxidants

Uric acid

Urate

Glutathione

Lipoic acid *

Dihydro-
Lipoic acid
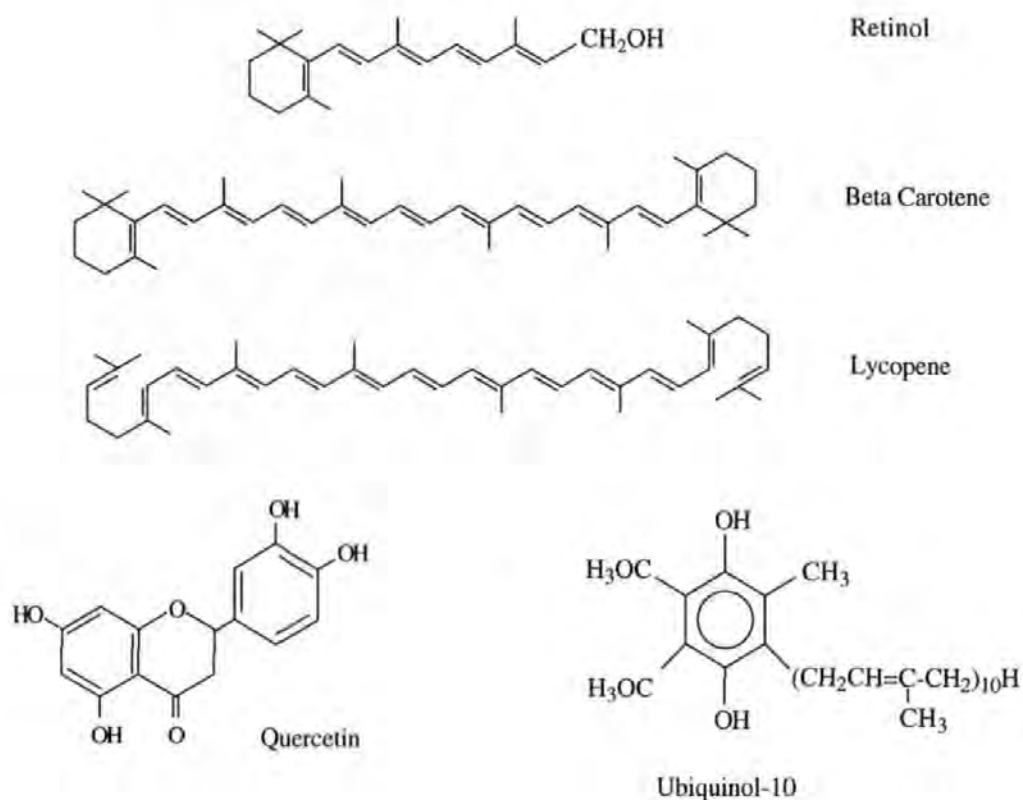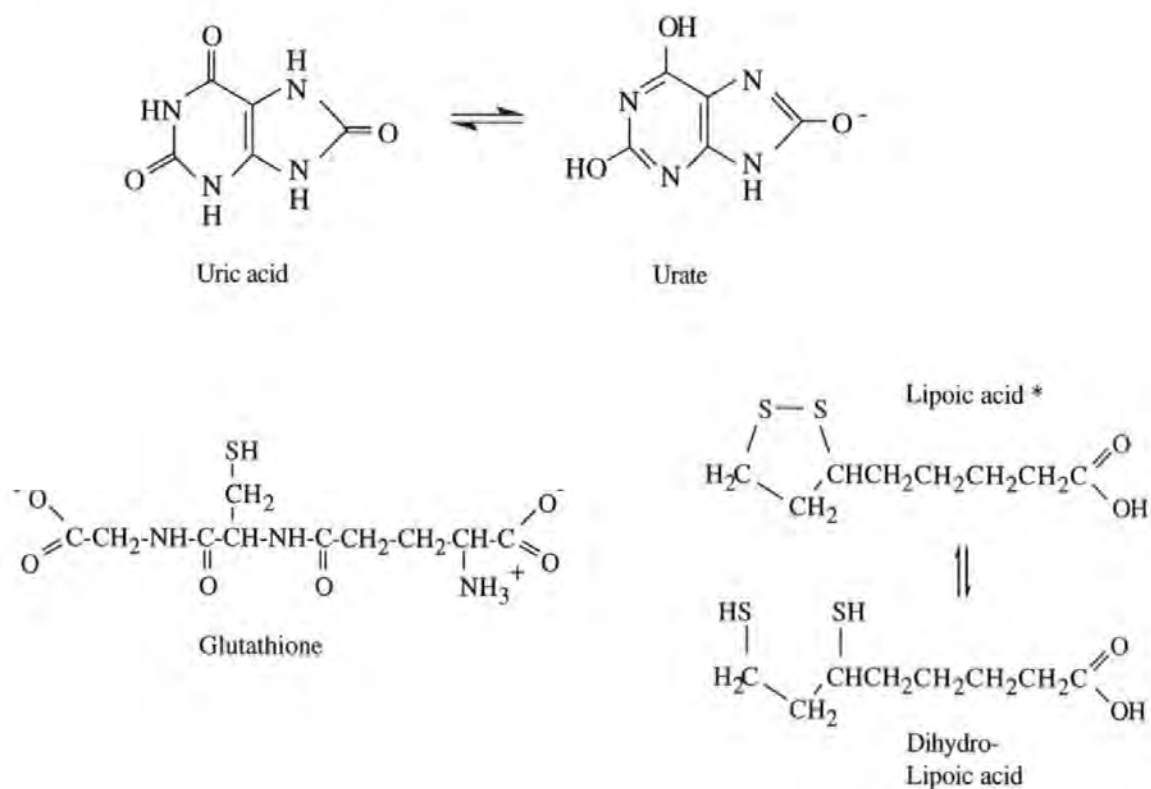
Figure 1.2 *Lipid and water soluble antioxidants.*
* *The lipoate redox couple functions in both lipid and aqueous domains.*

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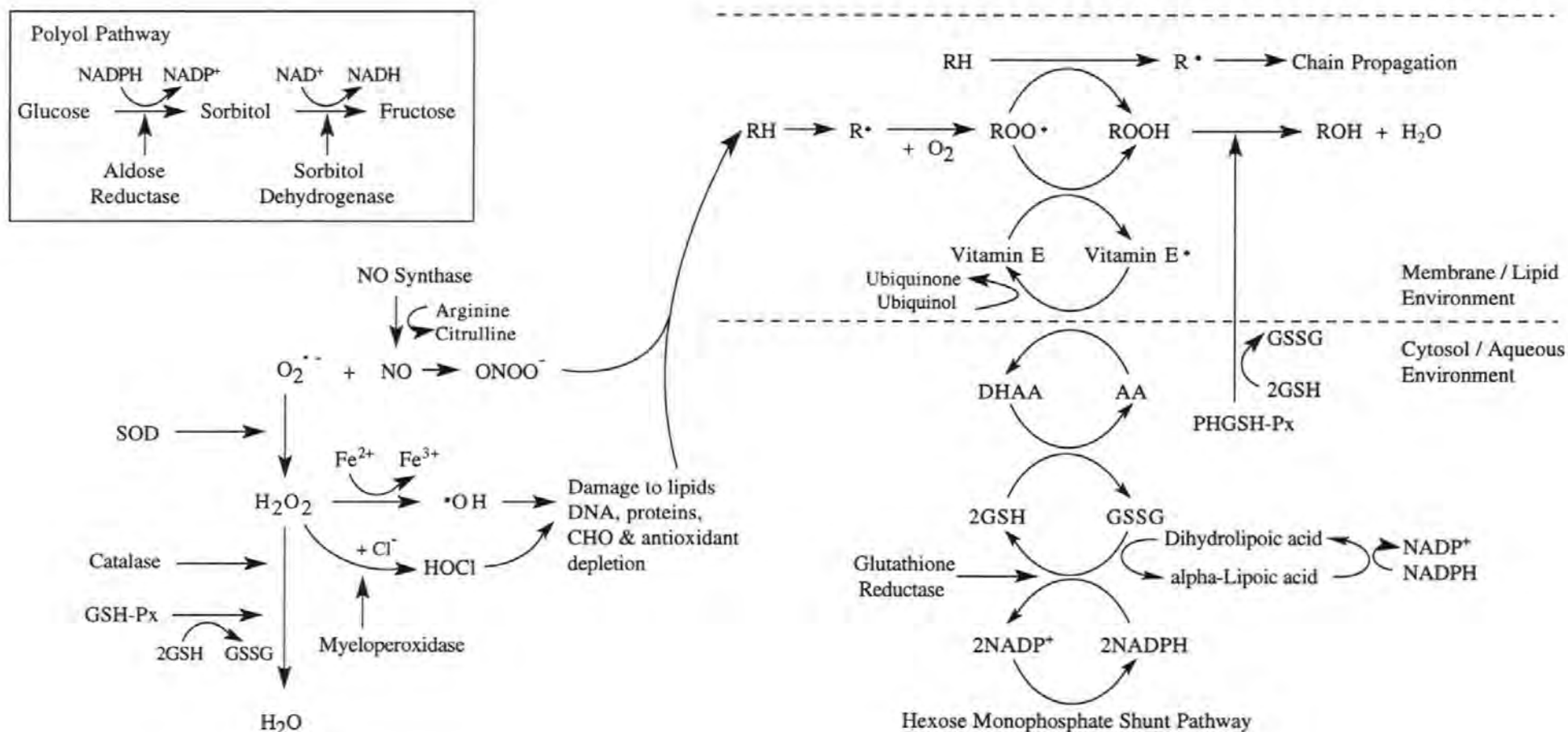
Figure 1.3 *Formation and removal of ROS and the interaction of antioxidants. Inset: the polyol pathway in diabetes, which may reduce the levels of NADPH required by glutathione reductases, resulting in the depletion of GSH, leading to oxidative stress.*

Ubiquinol

Although α-tocopherol is the most important lipid-soluble antioxidant, membranes also contain other antioxidants. The ubiquinols are lipid-soluble compounds which possess antioxidant activity (Mellors and Tappell 1966). The predominant form in man is ubiquinol-10, the reduced form of ubiquinone-10 or coenzyme Q10, an electron carrier between flavoproteins and cytochromes in the respiratory chain, Figure 1.2 (Frei *et al*. 1990). Ubiquinol-10 possesses antioxidant activity and has been reported to scavenge ROO· and $^1O_2$ (Frei *et al*. 1990; Stocker *et al*. 1991) and possibly regenerates the tocopheroxyl radical (Maguire *et al*. 1992; Frei *et al*. 1990; Kagan *et al*. 1990; Kagan *et al*. 1994). In fact, it appears that ubiquinol-10 is more effective than α-tocopherol in protecting LDL against oxidation (Stocker *et al*. 1991). However, LDL contains on average only one molecule of ubiquinol, therefore, α-tocopherol and ascorbate are the main antioxidants which suppress the oxidation of LDL in plasma (Ingold *et al*. 1993).

Carotenoids

The carotenoids are a group of approximately 600 photosynthetic plant pigments which also possess lipid-soluble antioxidant properties, on account of their characteristic chains of conjugated double bonds (Goodwin 1986; Krinsky 1993). Epidemiological evidence linking ß-carotene (provitamin A) intake with a reduced incidence of cancer (Peto *et al*. 1981), led to an explosion in the interest of carotenoids in cardiovascular disease and cancer (Gerster 1991; Block *et al*. 1992). Carotenoids provide protection against ROS, by quenching $^1O_2$ and triplet state sensitizers, such as flavins and porphyrins, and also by trapping peroxyl radicals (Palozza and Krinsky 1992b; Krinsky 1993).

The most prominent carotenoids in the body, ß-carotene, α-carotene, lutein and lycopene (Figure 1.2) are transported in plasma primarily within the LDL particles. ß-Carotene is known to be one of the most potent quenchers of $^1O_2$ (Foote and Denny 1968; Sundquist *et al*. 1994), especially at low partial pressures of O_2, such as those that prevail under physiological conditions (Burton and Ingold 1984). Several other carotenoids are also

effective quenchers of $^1O_2$, lycopene exhibiting the highest activity (Di Mascio *et al.* 1989; Sundquist *et al.* 1994). The quenching of $^1O_2$ occurs by energy transfer from $^1O_2$ to the carotenoid, yielding ground state O_2 and a triplet state carotenoid molecule, which dissipates the energy as heat so that no regeneration of the molecule is necessary. In addition to quenching $^1O_2$, *in vitro* studies indicate that ß-carotene acts as an efficient peroxyl radical scavenger, by enabling the covalent addition of peroxyl radicals to the conjugated chain (Burton and Ingold 1984; Burton 1989). ß-Carotene has also been shown to scavenge nitrogen dioxide (NO_2), thiyl ($RS\cdot$), and sulphonyl ($RSO_2\cdot$) radicals (Everett *et al.* 1996). A synergistic interaction between ß-carotene and α-tocopherol has also been observed which inhibits lipid peroxidation in microsomes (Palozza and Krinsky 1992a) and appears to prolong the lag phase in the oxidation of LDL (Esterbauer *et al.* 1992).

Vitamin A

Vitamin A, is a generic term for the fat-soluble ß-ionone ring compounds, which exhibit the biological activity of all-*trans*-retinol, Figure 1.2 (Pitt 1985). Vitamin A from dietary sources is stored in the liver esterified to fatty acids, primarily palmitic acid. For release into the plasma, vitamin A in the form of retinol, is bound to retinol binding protein (RBP), synthesized by the liver, for which zinc and an adequate intake of protein are required. Retinol resides within a hydrophobic fold of RBP, protecting it from oxidation during transport. Upon secretion into the plasma, RBP is complexed with another protein, transthyretin, to prevent loss through glomerular filtration (Olson 1991).

Retinol has a well defined role in the visual process and is important for cell growth, reproduction and maintaining the integrity of epithelial cells (Basu and Dickerson 1996). Additionally, retinol has been reported to possess antioxidant activity by scavenging peroxyl and thiyl radicals, thereby inhibiting lipid peroxidation (Halevy and Sklan 1987; D'Aquino *et al.* 1989; Hiramatsu and Packer 1990; Tsuchiya *et al.* 1992). The accumulation of retinol in membranes and LDL also resulted in increased resistance to oxidative stress, implying an antioxidant role (Ciacco *et al.* 1993; Livrea and Tesoriere 1994; Livrea *et al.* 1995).

(ii) Water-soluble antioxidants

Vitamin C

Vitamin C is an essential micronutrient for only a small number of species, including man and other primates, guinea pigs and certain fish, birds and bats. Deficiency results in the disease scurvy which can be life threatening (Counsell and Hornig 1981). Vitamin C occurs as L-ascorbic acid (AA) and L-dehydroascorbic acid (DHAA), both of which are biologically active.

Ascorbic acid acts as a physiological reducing agent by undergoing reversible oxidation to DHAA. At physiological pH, the ascorbate anion predominates. After the loss of one electron, the ascorbate free radical intermediate is formed (Bielski and Richter 1975), which yields DHAA after the loss of a second electron and proton (Figure 1.4). Dehydroascorbic acid undergoes irreversible hydrolysis to 2,3 diketogulonic acid, followed by oxidation to oxalic acid and threonic acid, which are excreted in the urine. However, intracellularly DHAA can be reduced back to AA by GSH, which in turn is linked with glutathione-reductase activity and the hexose monophosphate shunt pathway (Figure 1.3). The reduction of DHAA also appears to be mediated by DHAA-reductase activity, although no specific enzyme has yet been isolated in mammalian cells (Rose and Bode 1992).

Ascorbic acid has numerous diverse functions on account of its ability to provide enzymes with reducing equivalents (Levine 1986). For example, ascorbate acts as a cofactor in the biosynthesis of collagen, catecholamines, carnitine and is involved in the metabolism of amino acids, conversion of cholesterol to bile acids and detoxification of xenobiotics (Moser and Bendich 1991; Basu and Dickerson 1996).

The redox properties of AA make the vitamin an excellent antioxidant, capable of scavenging a wide range of ROS, including $O_2^{\cdot-}$, H_2O_2, $\cdot OH$, $HOCl$, $^1O_2$, $ONOO^-$ and aqueous $ROO\cdot$ radicals (Nishikimi 1975; Bodannes and Chan 1979; Cabelli and Bielski 1983; Bendich *et al.* 1986; Kwon and Foote 1988; Frei *et al.* 1989; Van der Vliet *et al.* 1994).

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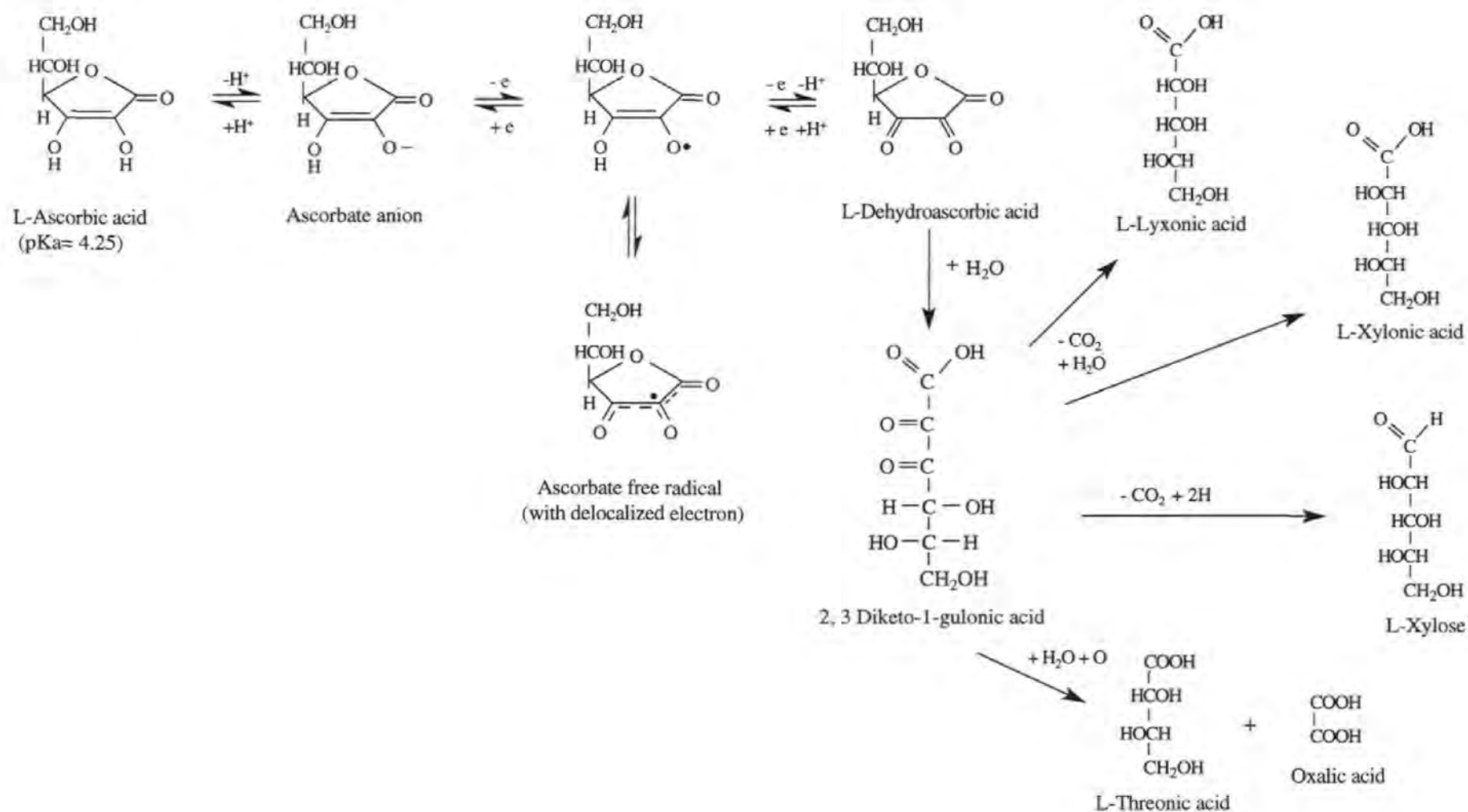
Figure 1.4 *Structure and oxidation of ascorbic acid.*

In studies with human plasma, AA was found to trap all aqueous peroxyl radicals before they could initiate lipid peroxidation. Once all the ascorbate had been consumed, the other aqueous antioxidants were found to trap only part of the free radicals and lipid peroxidation was initiated (Frei *et al.* 1988; Frei *et al.* 1989). The order of depletion of antioxidants was found to be AA, protein thiols, bilirubin, urate and α-tocopherol (Frei *et al.* 1989). By trapping aqueous radicals, AA protects membranes against peroxidative damage. Ascorbate also appears to protect membranes from lipid peroxidation by enhancing the activity of α-tocopherol (Sato *et al.* 1990). It is thought that AA may function to regenerate the tocopheroxyl radical (Figures 1.1 and 1.3), thereby increasing the scavenging potential of the antioxidant. Such a mechanism has been demonstrated with *in vitro* studies, but has yet to be demonstrated *in vivo* (McCay 1985). As a result of its scavenging ability, AA functions as a primary defence against aqueous radicals in plasma (Frei *et al.* 1989; Niki *et al.* 1991) and may act synergistically with tocopherols to protect LDL against oxidation (Sato *et al.* 1990; Ingold *et al.* 1993).

Ascorbic acid is also known to act as a pro-oxidant by reducing metal ions (e.g., Fe^{3+} to Fe^{2+}), which may then react with peroxides forming ·OH. These reactions have been used for the initiation of lipid peroxidation *in vitro* (Halliwell and Gutteridge 1990a). However, as there are effectively no free metal ions *in vivo* (Gutteridge *et al.* 1981a) the antioxidant properties of AA predominate (Frei 1994).

Uric acid

Uric acid originates from dietary sources and is also formed in the body by the oxidation of purines, via the intermediates hypoxanthine and xanthine, catalysed by xanthine dehydrogenase. At physiological pH, uric acid ionizes to urate (Figure 1.2). It has been suggested that the high urate concentrations in human plasma have contributed to the long lifespan of man, by acting as an important antioxidant (Ames *et al.* 1981; Cutler 1984; Becker 1993). This proposal has been supported by *in vitro* experiments which have demonstrated the scavenging properties of uric acid towards ·OH, ROO· and $^1O_2$ (Ames *et al.* 1981) and also guanyl radicals and NO_2 (Simic and Jovanovic 1989). Uric acid was

also found to inhibit lipid peroxidation of erythrocyte membranes (Ames *et al.* 1981) and LDL (Sato *et al.* 1990). Furthermore, uric acid appeared to have a protective role towards AA, by acting as a chelating agent and binding metal ions in forms that were poorly reactive (Davies *et al.* 1986). Ascorbate may also enhance the antioxidant ability of urate by regenerating the urate radical (Simic and Jovanovic 1989; Maples and Mason 1988).

Other molecules which may have important free radical scavenging roles include bilirubin and α-lipoic acid. Albumin-bound bilirubin appears to function as an important site-specific antioxidant by protecting albumin-bound fatty acids against peroxyl radical attack (Stocker *et al.* 1987; Stocker *et al.* 1990). The reduced form of α-lipoic acid (dihydrolipoic acid, Figure 1.2), an acyl-group carrier and an essential cofactor in mitochondrial dehydrogenase reactions, also scavenges ROS and regenerates other antioxidants by redox cycling, Figure 1.3 (Suzuki *et al.* 1994; Packer *et al.* 1995; Packer *et al.* 1997). Furthermore, α-lipoic acid supplementation has been found to produce several beneficial effects in the treatment of diabetes (Nickander *et al.* 1996; Ziegler *et al.* 1997).

In addition to the antioxidants listed in Table 1.4, attention is focussing on other compounds of plant origin which have antioxidant properties and which may contribute to human health (Aruoma *et al.* 1996; Kumpulainen and Salonen 1996; Rice-Evans and Miller 1996). The flavonoid compounds are a group of over 4000 polyphenolic pigments, ubiquitous in plants, which have received much attention (Das 1994; van Acker *et al.* 1996; Cao *et al.* 1997). These compounds have been found to scavenge free radicals (Husain *et al.* 1987; Robak and Gryglewski 1988; Bors *et al.* 1994), inhibit lipid peroxidation (Afanas'ev *et al.* 1989; Yuting *et al.* 1990) and increase the resistance of LDL to oxidation (De Whally *et al.* 1990; Fuhrman 1995; Vinson *et al.* 1995). Hence, diets rich in flavonoids may be protective against CHD (Hertog *et al.* 1993; Hertog *et al.* 1995; Knekt *et al.* 1996). One of the most studied flavonoids, quercetin (Figure 1.2), was also found to inhibit the action of many enzymes, including aldose reductase (Varma and Kinoshita 1976). Interest is also increasing in the role of other compounds, including the polyphenols found in beverages and the extracts of herbs and spices (Graf *et al.* 1987; Ramarathnam *et al.* 1995; Aruoma *et al.* 1996).

1.3   Lipid Peroxidation

The possibilities for the formation of free radicals abound in biological systems. However, their formation is usually only transient and they are compartmentalized within cells and effectively 'insulated' from interaction with other radicals by antioxidants and scavenger systems. If cellular damage occurs, or the balance between the formation and removal of free radicals is altered, enabling the interaction of different ROS and metal ions, highly reactive radicals such as ·OH can be formed. The formation of such highly reactive molecules can result in damage to any molecule within the immediate vicinity. Attack upon lipids can result in lipid peroxidation.

Lipid peroxidation proceeds by an autocatalytic, non-enzymatic, free radical chain reaction, yielding lipid hydroperoxides as the principal products (Farmer *et al.* 1942; Bateman 1954). The effects of lipid peroxidation are highly undesirable and the hydroperoxides produced may be subsequently converted into a wide variety of low molecular-weight degradation products, which have implications in many fields from food science through to clinical medicine (Chan 1987). In common with other free radical reactions, lipid peroxidation can be divided into three processes: initiation, propagation and termination (Porter *et al.* 1995).

1.3.1   Initiation

In a peroxide-free lipid system, initiation refers to the initial attack of an unsaturated fatty acid (RH), by a free radical (X·), with sufficient reactivity to abstract a hydrogen atom from a methylene group ($-CH_2-$), reaction 1.13.

Initiation: X· + RH → R· + XH   (1.13)
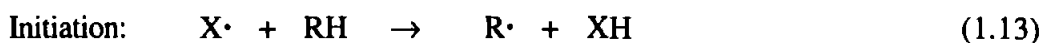
The presence of a double bond adjacent to a methylene group causes a weakening of the C-H bond, making the removal of the hydrogen easier. As the number of double bonds increases, the removal of hydrogen is made even easier. Thus, PUFAs, such as arachidonic acid in membranes, are particularly susceptible to free radical attack (Cosgrove *et al.* 1987).

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The exact nature of the radicals initiating lipid peroxidation *in vivo* remains an unresolved issue. The superoxide radical is insufficiently reactive to abstract a hydrogen atom and under normal conditions would be scavenged by SOD, forming H_2O_2, which in turn would be scavenged by catalase and GSH-Px. The protonated form of $O_2^{\cdot-}$, the hydroperoxyl radical ($HO_2^{\cdot}$), is more reactive and can initiate lipid peroxidation *in vitro* (Bielski *et al.* 1983; Aikens and Dix 1991), but at physiological pH dissociates to $O_2^{\cdot-}$ (Gutteridge 1995).

The highly reactive hydroxyl radical is regarded as being responsible for initiating lipid peroxidation, because if it is formed in close proximity to a fatty acid molecule, it will readily abstract a hydrogen atom (Minotti and Aust 1987a). The decomposition of H_2O_2 can also result in the production of $\cdot OH$ (reactions 1.8-1.10). However, studies of lipid peroxidation involving the addition of iron salts to isolated membranes, such as microsomes and liposomes, have indicated that the importance of $\cdot OH$ may have been over emphasized. Hydroxyl radicals were detected in such systems, but the addition of catalase to remove H_2O_2 and scavengers for $\cdot OH$ did not inhibit the peroxidation process (Gutteridge 1982; Halliwell and Gutteridge 1984; Minotti and Aust 1987b; Halliwell and Gutteridge 1990a). This led to the suggestion that $\cdot OH$ was not required for peroxidation to proceed and that first chain initiation in membrane systems was achieved by reactive species other than $\cdot OH$, such as iron / iron-oxygen complexes (Minotti and Aust 1987a; Halliwell and Gutteridge 1990a). One possibility that perferryl complexes were responsible for initiation was diminished by the finding that other metal ions, such as aluminium and lead, could also stimulate lipid peroxidation (Aruoma *et al.* 1989). However, once peroxidation is underway, the metal catalysed decomposition of lipid hydroperoxides can produce radicals which are capable of initiating further reactions (reactions 1.17 and 1.18) (Gutteridge 1995).

In the cellular environment, iron is tightly sequestered in iron complexes such as ferritin and haemosiderin and is unavailable to participate in redox reactions, unless it is released in a free form, e.g., after cellular damage. It may be the case that membrane-bound iron ions are responsible for 'site-specific' formation of free radicals (Halliwell and Gutteridge 1989).

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The hydroxyl radical is commonly assumed to be the most toxic of oxygen radicals and mediator of oxygen toxicity. However, attention is now focussing on the role of other compounds which are capable of initiating lipid peroxidation, such as thiyl radicals and peroxynitrite (Schöneich *et al.* 1989; Beckman *et al.* 1990). Indeed, the oxidative chemistry of NO and related compounds is an area of intensive research. The combination of $O_2^{\cdot-}$ and NO results in the formation of peroxynitrite, a strong oxidant capable of producing species with reactivity comparable to ·OH (Beckman *et al.* 1990). Peroxynitrite can be formed *in vivo* and is capable of inducing lipid peroxidation, reactions which do not require the presence of transition metal ions (Radi *et al.* 1991a; Rubbo *et al.* 1994).

The addition of singlet oxygen to unsaturated fatty acids may also be responsible for the first formed hydroperoxides (Rawls and van Santen 1970). In practice it is difficult to obtain fatty acids which are free from peroxides, as even commercially available standards are contaminated with traces of peroxides. Thus, even in the absence of initiators, lipid peroxidation may proceed with a very slow induction period until the levels of hydroperoxides build up (Gunstone 1996).

1.3.2 Propagation

Propagation:
$$R\cdot \quad + \ RH \quad \rightarrow \quad RH \ + \ R\cdot \quad\quad (1.14)$$
$$R\cdot \quad + \ O_2 \quad \rightarrow \quad ROO\cdot \quad\quad\quad\quad (1.15)$$
$$ROO\cdot \ + \ RH \quad \rightarrow \quad ROOH \ + \ R\cdot \quad\quad (1.16)$$
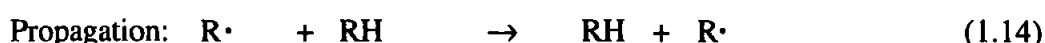
The abstraction of hydrogen from a $-CH_2-$ group leaves behind an unpaired electron on the carbon ($-\cdot CH-$). The resulting carbon radical becomes stabilized by molecular rearrangement with the formation of a conjugated diene (Figures 1.5 and 1.6, pages 31 and 32). The reactions of the conjugated dienes depend on the concentration of oxygen. At low oxygen concentrations, one conjugated diene may react with another conjugated diene (reaction 1.19) or a protein forming a crosslink between two molecules, or abstract a

hydrogen from an adjacent fatty acid creating another carbon-centred radical (reaction 1.14). Under aerobic conditions, the most likely event is the combination with molecular oxygen, forming a peroxyl radical (ROO·), reaction 1.15. The peroxyl radical is capable of abstracting a hydrogen atom from an adjacent unsaturated fatty acid, forming a lipid hydroperoxide (ROOH) and another lipid radical, thereby propagating the reaction (reaction 1.16). The propagation reactions form the basis of the chain reaction process characteristic of free radical activity and may continue until a termination reaction occurs.

The lipid hydroperoxides are stable intermediates, but the presence of transition metals, especially iron, can catalyse their decomposition via the formation of perferryl complexes, in reactions similar to the decomposition of H_2O_2 (reaction 1.9). The decomposition leads to the production of alkoxyl (RO·) or peroxyl (ROO·) radicals, the overall reactions are shown in 1.17 and 1.18. Alkoxyl radicals have very short half-lives (Table 1.1) and will attack proteins or lipids, thereby initiating further chain reactions (Halliwell and Gutteridge 1989).

$$ROOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^- + RO· \qquad (1.17)$$

$$ROOH + Fe^{3+}\text{-complex} \rightarrow Fe^{2+}\text{-complex} + H^+ + ROO· \qquad (1.18)$$

1.3.3   Termination

Free radical chain reactions are terminated by the combination of two radicals which form a non-radical product (reactions 1.19 - 1.23).

Termination:	R·	+	R·	→	R-R		(1.19)
	ROO·	+	ROO·	→	PRODUCTS		(1.20)
	ROO·	+	R·	→	ROOR		(1.21)
	R·	+	AH	→	RH	+ A·	(1.22)
	ROO·	+	AH	→	ROOH	+ A·	(1.23)
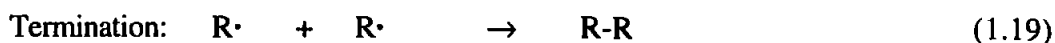
The chain reactions may also be terminated by antioxidants (AH), primarily vitamin E in membranes, which donate hydrogen atoms to the fatty acid radicals, in order to minimize the number of propagation cycles (reactions 1.22 and 1.23). The resulting antioxidant free radicals form stable intermediates in a biological matrix and can be 'recycled' by other molecules. Vitamin C or glutathione are believed to regenerate the vitamin E radical *in vivo* (McCay 1985).

The termination reaction between two lipid peroxyl radicals (reaction 1.20) proceeds via the formation of an unstable tetroxide (the Russell mechanism, reaction 1.24), which decomposes to a ketone with an excited triplet state carbonyl group (^3RO), an alcohol and oxygen (Russell 1957; Kellogg 1969). The quenching of the excited carbonyl group by oxygen results in the formation of $^1O_2$, reaction 1.25. Howard and Ingold (1968) identified $^1O_2$ in the self-reaction of butyl peroxyl radicals, supporting the Russell mechanism of termination.

$$2\ ROO^{\cdot} \leftrightarrow ROOOOR \rightarrow {}^3RO + ROH + O_2 \qquad (1.24)$$

$$^3RO + O_2 \rightarrow RO + {}^1O_2 \qquad (1.25)$$
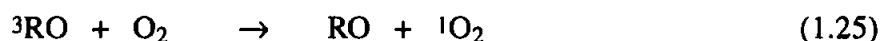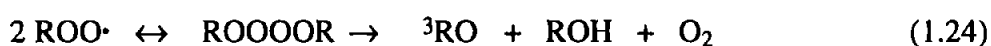
Chemiluminescence has indicated the presence of $^1O_2$ in biological systems, where its formation was attributed to phagocytosis, photosensitization reactions and lipid peroxidation (Foote *et al.* 1984; Cadenas and Sies 1984; Murphy and Sies 1990). Singlet oxygen will readily add to a double bond in an unsaturated fatty acid forming a hydroperoxide. Although the reaction does not proceed via the formation of free radical intermediates, the subsequent decomposition of the hydroperoxides can produce free radicals (Gunstone 1996). The role of lipid hydroperoxides in cellular damage is, therefore, of importance as they are minor constituents of membranes and their subsequent decomposition may be the cause of cellular lipid peroxidation (Ursini *et al.* 1991).

1.3.4 Decomposition of lipid hydroperoxides

Lipid hydroperoxides may undergo a variety of reactions, including polymerization reactions resulting in intermolecular crosslinks, or rearrangement reactions forming cyclic peroxides and cyclic endoperoxides Figure 1.6. As shown in reaction 1.17, lipid hydroperoxide decomposition can result in the formation of alkoxyl radicals. These may undergo ß-scission of the carbon-carbon bond adjacent to the hydroperoxide group (Figure 1.5), and depending on the position of the peroxide group on the fatty acid chain, a complex mixture of secondary products of lipid peroxidation can be formed. In summary, the main volatile products include aldehydes such as alkanals, 2 alkenals, 2,4 alkadienals. The major aldehydes in biological samples appear to be malondialdehyde (MDA), hexanal and 4-hydroxynonenal (Zollner *et al.* 1991). Other compounds include hydroxyacids, ketoacids, ketones alcohols and hydrocarbons (Esterbauer 1982a; Esterbauer 1982b).

Several secondary products are themselves reactive and have been shown to react with cellular components, 4-hydroxynonenal is cytotoxic and mutagenic (Zollner *et al.* 1991; Esterbauer 1993). Malondialdehyde reacts with amino groups in proteins forming fluorescent crosslinks —aminoiminopropene Schiff bases (Dillard and Tappel 1984).

Linoleic acid

Initiation − H•

Hydrogen abstraction

Carbon-centred radical

Molecular rearrangement resulting in the formation of a conjugated diene with UV absorbance at 234nm.

+ O₂

Oxygen uptake with the formation of a peroxyl radical.

Propagation RH AH Termination
R• A•

Abstraction of hydrogen from another fatty acid (RH) propagating the chain reaction, or termination by an antioxidant (AH).

Lipid hydroperoxide

Fe^{2+}

Fe^{3+}

Metal catalysed decomposition of the hydroperoxide to the alkoxyl radical, which can initiate further free radical reactions or undergo cleavage of the C-C bond forming an aldehyde and a pentyl radical.

Beta-scission

* R= $(CH_2)_4COOH$

Figure 1.5 *Lipid peroxidation of linoleic acid, with the formation of hydroperoxides and secondary breakdown products.*

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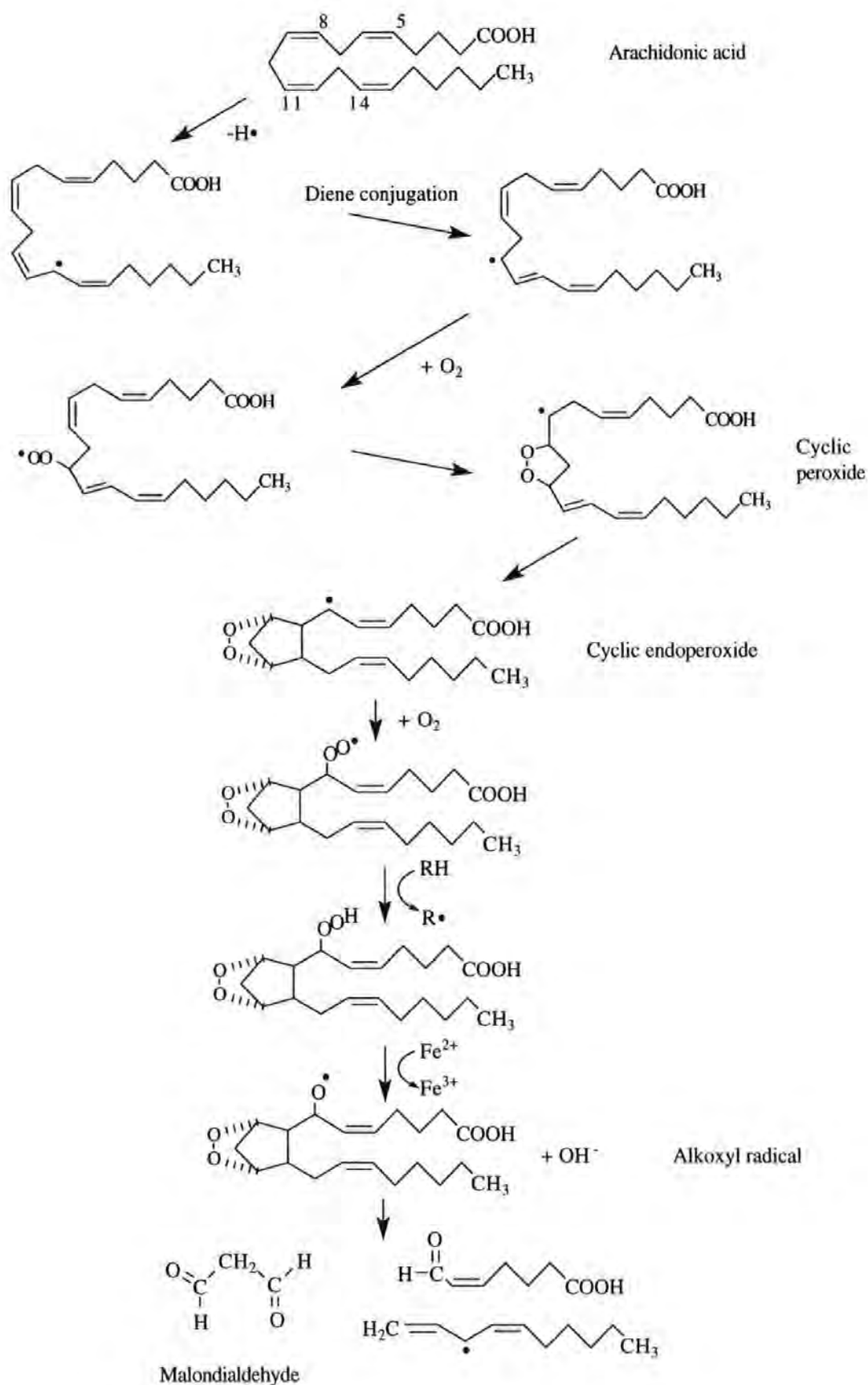
Figure 1.6 *Lipid peroxidation of arachidonic acid (modified from Aruoma and Halliwell 1991).*

PUFA   Loss of substrate, GC or HPLC analysis

| -H•

Free radicals   ESR, spin trapping

| Molecular   UV absorbance of conjugated dienes
| rearrangement

| + O$_2$ O$_2$ concentration

Lipid peroxides   Iodine liberation
                               Oxidation of Fe^{2+} to Fe^{3+}
                               Isoluminol-Chemiluminescence

Cyclic endoperoxides   HPLC, GC-MS
                               GC-MS of Prostaglandin-like
| compounds (isoprostanes)
| Decomposition
|

Excited carbonyls + ^{1}O$_2$ Chemiluminescence

Alkanes, alkenes   GC

Alkanals, e.g., malondialdehyde   TBARS

Alkenals, e.g., 4 hydroxynonenal   TLC, GC-MS, HPLC

|
|

Reactions with proteins   Fluorescence detection of Schiff bases,
                               Antibody techniques
                               ELISA

Figure 1.7 *Methods used for the detection and measurement of the different stages of lipid peroxidation. ESR, electron spin resonance; GC, gas chromatography; GC-MS, gas chromatography-mass spectrometry; HPLC, high performance liquid chromatography; TBARS, thiobarbituric acid reactive substances; TLC, thin layer chromatography; UV ultra violet; ELISA, enzyme linked immunosorbent assay.*

1.3.5   Measurement of lipid peroxidation

The detection and measurement of products of lipid peroxidation is the evidence most frequently used to provide an indirect indication of free radical activity in human disease. Numerous methods, outlined in Figure 1.7, are available for the measurement of the different stages of lipid peroxidation (reviewed by Slater 1984b; Rice-Evans *et al.* 1991; Halliwell and Chirico 1993; Gutteridge 1995; Esterbauer 1996; Punchard and Kelly 1996); although, no single technique is adequate for all stages of the process in a biological setting. The two most frequently used assays, the measurement of conjugated dienes and the thiobarbituric acid (TBA) test, are the main interest in this thesis.

(i)   Conjugated dienes

During lipid peroxidation, a shift in the location of a double bond leading to the formation of a conjugated diene is accompanied by an increase in UV absorbance, in the wavelength range 230-235nm. In studies of pure lipids, the increase in absorbance provided a good indication of the early stages of lipid peroxidation, as demonstrated by the pioneering work of Farmer and Sutton (1943). This procedure is still widely used for monitoring the oxidation of LDL *in vitro* (Esterbauer *et al.* 1991; Esterbauer *et al.* 1992).

In biological samples, the presence of numerous substances with UV absorbing properties indicated the need for additional techniques. Extraction of the lipids prior to analysis and second derivative spectroscopy have been used to increase the sensitivity for the measurement of conjugated dienes in plasma (Recknagel and Glende 1984; Situnayake *et al.* 1990). High performance liquid chromatography (HPLC) has also been used to separate the conjugated dienes in plasma from interfering compounds (Cawood *et al.* 1983). The extensive studies of Iversen *et al.* (1984) have demonstrated that in fresh human serum and tissue fluids, diene conjugation is predominantly attributed to a single fatty acid, octadeca,9-*cis*,11-*trans* dienoic acid (18:2(9-*cis*, 11-*trans*)), which contains no other oxygen than that of the carboxyl group (Figure 1.5). The generation of this isomer from linoleic acid (18:2(9-*cis*,12-*cis*)) is thought to require the interaction of the carbon-centred radical with a protein reducing group, such as a thiol residue (Cawood *et al.* 1983; Iversen *et al.* 1984).

There is, however, a degree of uncertainty regarding the origin of the isomer in biological fluids. Thompson and Smith (1985) measured the concentration of 18:2(9-*cis*, 11-*trans*) in human and rat blood, after the *in vitro* initiation of lipid peroxidation by UV irradiation or phenylhydrazine treatment, and also *in vivo* in rats pretreated with bromotrichloromethane or phenylhydrazine. Plasma levels of total conjugated dienes were significantly increased in both studies, but the levels of the conjugated diene isomer of linoleic acid showed little change. It was concluded that this conjugated diene was a poor indicator of free radical activity in animal studies.

The stereo specificity of the 18:2(9-*cis*, 11-*trans*) isomer has been the principal reason for the doubt concerning its free radical origin. Such specificity would suggest that an underlying enzymatic mechanism might be responsible for its formation (Iversen *et al.* 1984). Indeed, certain bacteria and rumen microorganisms have been reported to generate the isomer from linoleic acid (Hughes *et al.* 1982; Fairbank *et al.* 1988). Plasma levels in humans may, therefore, reflect dietary intake of dairy products (Thompson and Smith 1985; Wickens and Dormandy 1988). Although, it may also be possible that a free radical mechanism, taking place in a structured membrane system, may result in the formation of a stereo specific isomer (Iversen *et al.* 1984; Wickens and Dormandy 1988).

Despite the doubt surrounding the validity of the 18:2(9-*cis*, 11-*trans*) isomer as a marker of free radical mediated lipid peroxidation, significant increases in the concentration of the isomer were detected in human pathologies associated with increased free radical activity, such as paraquat poisoning, which is known to involve the formation of free radicals (Yasaka *et al.* 1981; Crump *et al.* 1985; Stohs 1995). Hence, the finding of elevated levels of conjugated dienes in the plasma of diabetic patients, added support to the hypothesis that free radical activity and oxidative stress are increased in diabetes (Section 1.4.2 and Appendix 3).

(ii) Thiobarbituric acid test

The TBA test is one of the most frequently used methods for the measurement of MDA and lipid peroxides in fatty acids, food products, membrane systems, plasma and tissue samples (Halliwell and Gutteridge 1990a; Draper and Hadley 1990). The general procedure simply involves heating the material under test with TBA in acidic conditions and measuring the absorption of the resulting pink chromogen, either spectrophotometrically or fluorimetrically. The chromogen, which forms by the condensation of two molecules of TBA and one molecule of MDA, absorbs light at 532nm and fluoresces at 553nm (Sinnhuber et al. 1958). The adduct was also characterized as having two tautomeric isomers (Nair and Turner 1984).

Malondialdehyde is formed from fatty acids containing at least three double bonds, particularly arachidonic acid (McBrien and Slater 1982). Small amounts of free MDA are generated during the peroxidation process itself. The remainder arises from the decomposition of cyclic endoperoxides during the acid heating stage of the test (Pryor et al. 1976; Wong et al. 1987). Hence the term 'thiobarbituric acid reactive substances' (TBARS), has also been applied to the test (Draper and Hadley 1990).

Yagi et al. (1968) first applied the TBA reaction for the determination of lipid peroxides in serum and numerous techniques are variations of the fluorimetric procedure published by Yagi in 1976 (Hackett et al. 1988; Kojima et al. 1990; Conti et al. 1991; Richard et al. 1992; Agil et al. 1995). The lack of specificity has been the main criticism of the test, as numerous compounds present in biological samples, including carbohydrates, amino acids, unsaturated aldehydes and bile pigments react with TBA and could cause interference in spectro-photometric and fluorimetric procedures (Esterbauer et al. 1982b; Knight et al. 1988; Kojima et al. 1990). For this reason HPLC has been used to separate the real TBA-MDA adduct from the contaminants prior to measurement, thereby increasing the specificity of the assay (Bird et al. 1983; Wade et al. 1985; Wong et al. 1987; Carbonneau et al. 1991; Lepage et al. 1991; Young and Trimble 1991). However, it must be noted that several carbohydrates, including sucrose and deoxyribose when exposed to ·OH radicals, produced by γ-radiolysis

of water or exposure to iron salts, yielded MDA and produced a true TBA-MDA adduct (Halliwell and Gutteridge 1981; Cheeseman *et al.* 1988). Such conditions are unlikely to occur *in vivo* (Draper and Hadley 1990).

Although the TBA test for MDA has the advantage of simplicity, some problems still remain. The colour developed in the reaction is affected by the reaction temperature and the heating time. Hence, the measured MDA value can vary a great deal (Hackett *et al.* 1988). The concentration of MDA in biological samples is, therefore, greatly influenced by the conditions employed in the assay. This is reflected by the wide variation in published values for the MDA concentration in the plasma of healthy subjects, as indicated in Table 1.5, making interlaboratory comparisons difficult.

The lack of specificity has led to the continued refinement of existing methods in order to minimize spectral interferences (Espinosa-Mansilla *et al.* 1993; Chirico 1994) and optimize the reaction conditions (Wade and van Rij 1988; Lepage *et al.* 1991). Despite the criticisms, the TBA test remains a popular assay. Using this assay Sato *et al.* (1979) were the first to report elevated levels of lipid peroxides in the serum of diabetic subjects.

Table 1.5 *Reported values for the MDA concentration in the plasma or serum of healthy subjects.*

Reference	Method	Plasma/ Serum	MDA concentration [a] μmol/l (mean±SD)
Sato *et al.* 1979	F [b]	plasma	3.74±0.13
Santos *et al.* 1980	S	plasma	35.1±5.9
Yasaka *et al.* 1981	F	plasma	3.74±0.63
Ledwozyw *et al.* 1986	S	plasma	0.94±0.09
Wong *et al.* 1987	HPLC+S	plasma	0.60±0.13
Young & Trimble 1991	HPLC+F	plasma	0.59±0.16
Carbonneau *et al.* 1991	HPLC+S	plasma	0.429±0.048
Carbonneau *et al.* 1991	HPLC+S	serum	0.454±0.066
Richard *et al.* 1992	F	plasma	2.51±0.25

[a] MDA concentration determined as the MDA-TBA adduct.
[b] S = UV Spectrophotometry; F = Fluorimetry; HPLC, High performance liquid chromatography.

1.3.6   Consequences of lipid peroxidation

The susceptibility of membrane lipids to peroxidation is largely influenced by their degree of unsaturation. Phospholipids contain a high proportion of PUFAs making cell membranes particularly susceptible to free radical attack. The consequences of lipid peroxidation in membranes include, decreased membrane fluidity and increased membrane permeability, which in turn lead to loss of function, impairment of membrane-bound enzymes and eventual rupture and release of contents (Niki *et al*. 1991). The secondary products of lipid peroxidation are themselves cytotoxic and capable of altering protein structure and enzyme activities (Zollner *et al*. 1991; Esterbauer 1993). Hence the products of lipid peroxidation, whether produced endogenously or ingested with food, are potentially detrimental to human health (Kubow 1990; Aruoma and Halliwell 1991; Esterbauer 1993).

There is confusion regarding the role of free radical reactions and lipid peroxidation in human toxicology and disease. Free radical activity has been implicated in the process of ageing and in numerous disease states, including diabetes mellitus. It is not always easy to ascertain whether free radical activity resulting in lipid peroxidation is the cause of the disease process or arises as a consequence of the tissue damage. However, lipid peroxidation does appear to make a significant contribution to the oxidative modification of LDL, a key stage in the development of atherosclerosis and CHD.

(i)   Atherosclerosis

Serum cholesterol is a major risk factor for CHD and growing evidence indicates that this risk is mediated through the major cholesterol carrying lipoprotein, the LDL (Grundy 1995; Berliner and Heinecke 1996). Lipid peroxidation of LDL, within the intima of the arterial wall, leads to the formation of reactive aldehydes which bind to the apoprotein B-100. Modification of this apoprotein triggers the recognition of these particles by the macrophage scavenger-receptor, resulting in the unregulated uptake of LDL and subsequent formation of foam cells (Goldstein *et al*. 1979b; Steinberg *et al*. 1989). As foam cells accumulate, they form fatty streaks, the first visible lesions in atherosclerosis. Proliferation of smooth muscle

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cells leads to the formation of fibrous plaques. If the endothelium is damaged, platelet accumulation may lead to thrombosis and vessel occlusion (Ross 1993; Schwartz and Valente 1994).

Several lines of investigation, from both *in vitro* and *in vivo* studies, have provided strong evidence that oxidative modification of LDL occurs during the development of fatty streaks (reviewed by Rice-Evans 1993; Keaney and Frei 1994; Jialal and Devaraj 1996; Reaven and Witztum 1996). Briefly, certain cells, including macrophages, smooth muscle cells and endothelial cells have been shown to modify LDL, stimulating uptake by the macrophage scavenger-receptor, *in vitro* (Henriksen *et al*. 1981; Steinbrecher *et al*. 1984; Leake and Rankin 1990) and *in vivo* (Palinski *et al*. 1989). The secondary products of lipid peroxidation can modify the apoproteins (Steinbrecher 1987), damage the endothelium and stimulate the infiltration of monocytes by chemoattraction, promoting plaque progression (Quinn *et al*. 1987). Antibodies to oxidized LDL have been demonstrated in both human and animal atherosclerotic lesions, but not in normal arteries (Palinski *et al*. 1989; Salonen *et al*. 1992). The contents of human atherosclerotic lesions were also found to stimulate lipid peroxidation (Smith *et al*. 1992). Furthermore, the extensive studies of Esterbauer *et al*. (1992) have shown that LDL contains a number of endogenous antioxidants and that it is only when these have been largely consumed that peroxidation takes place. Consistent with this is epidemiological evidence, that in populations with high dietary intakes of fruit and vegetables and high plasma levels of antioxidants, particularly vitamin E, the incidence of CHD is low (Gey and Puska 1989; Gey *et al*. 1991; Rimm *et al*. 1993; Stampfer *et al*. 1993; reviewed by Graziano *et al*. 1994). Recently, a randomized trial of vitamin E supplementation, in patients with angiographically confirmed atherosclerosis, has found a significant reduction in the risk of myocardial infarction after 1 year of treatment, supporting the lipid oxidation theory of atherosclerosis and preventative role of antioxidants in CHD (Stephens *et al*. 1996). A schematic representation of the role of oxidized LDL in atherogenesis is shown in Figure 1.8.
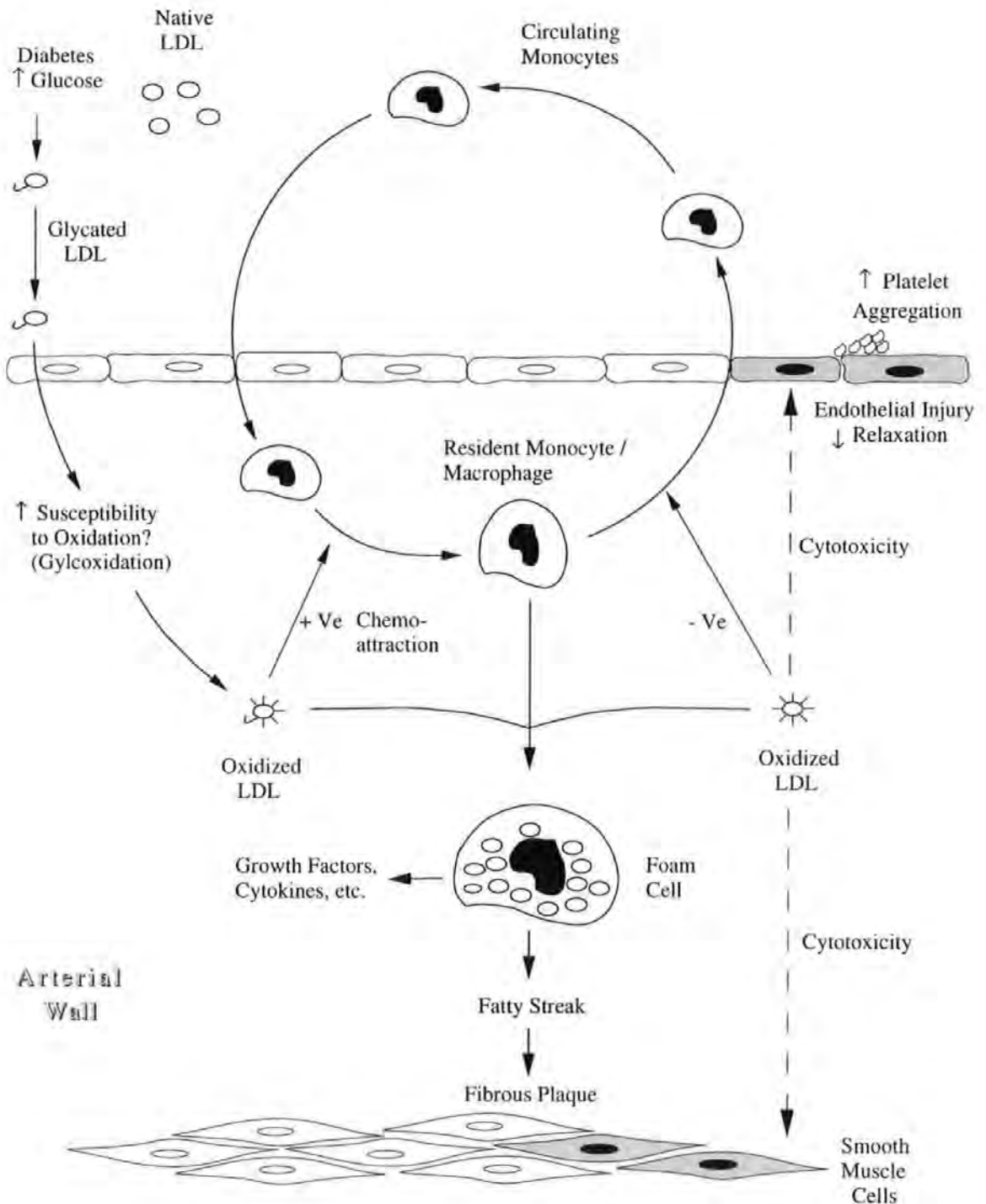
Figure 1.8 *Schematic representation of the role of LDL in atherosclerosis (modified from Quinn et al. 1987; Lyons 1991; Lyons 1993). Oxidized LDL is chemotactic (+Ve) for circulating monocytes, inhibiting their mobility and exit from the vessel wall (-Ve). Oxidized LDL is cytotoxic and also induces the release of a number of factors from macrophages and endothelial cells, which are chemotactic for monocytes, stimulate growth and adversely affect the coagulation pathway. ↑, increase; ↓, decrease.*

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1.4 Diabetes Mellitus

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia, secondary to an absolute or relative lack of insulin, or resistance to its action, which results in derangements in carbohydrate, lipid and protein metabolism. The diagnostic criteria for diabetes have been re-examined in recent years and the American Diabetes Association have recommended that the fasting plasma glucose level considered to be diagnostic for diabetes be lowered from 7.8 mmol/l (World Health Organization 1985) to 7.0 mmol/l (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997). Clinically, diabetes is subdivided into two main categories: insulin-dependent, or type I diabetes and non-insulin-dependent, or type II diabetes. Type I diabetes occurs mainly in people below 30 years of age, as a result of the autoimmune destruction of the pancreatic ß-cells and requires insulin replacement by daily injections. Omission of insulin results in diabetic ketoacidosis. Type II diabetes is a complex polygenic condition, which involves defects in both insulin secretion and insulin action. It is seldom seen in persons below 35 years of age and is often associated with obesity. The majority of patients fall into the type II category and are treated by diet, or diet and hypoglycaemic drug therapy although some patients require insulin.

Type I and type II diabetes are distinct conditions, which share a striking propensity to both microvascular and macrovascular disease, suggesting a common underlying cause. The microvascular complications, retinopathy, neuropathy and nephropathy are accepted to be primarily the result of the exposure of the tissues to the chronically elevated glucose levels, since the risk of developing complications increases with increasing duration of diabetes (Pirart 1978) and also with increasing hyperglycaemia (West 1982). Recently, the Diabetes Control and Complications Trial (DCCT, 1993) convincingly demonstrated that improved glycaemic control significantly delayed the onset and progression of complications, supporting the hypothesis that hyperglycaemia is the cause of microvascular complications in type I diabetes. However, the confounding factor is why patients with similar duration and degree of glycaemic control, differ markedly in their susceptibility to tissue damage (Raskin and Rosenstock 1986).

A small proportion (5%) of patients with type I diabetes appear to be particularly susceptible to developing microvascular complications, even with mild elevations in plasma glucose, whilst approximately 20% never develop severe complications, regardless of glycaemic control. The majority of patients, however, have varying degrees of susceptibility to complications. Consequently, two main hypotheses have been put forward to explain the widespread differences in the occurrence of microvascular complications: the genetic theory, which suggests that the microvascular complications are a genetically predetermined part of the diabetic syndrome and the metabolic theory, that the complications are solely sequelae of the hyperglycaemia (Raskin and Rosenstock 1992).

The metabolic pathways by which hyperglycaemia leads to tissue damage are areas of intensive research and several mechanisms have been proposed. These include: capillary basement membrane thickening, non-enzymatic glycosylation of proteins, increased flux through the polyol pathway and pseudohypoxia, haemodynamic changes and increased free radical activity / oxidative stress (Greene et al. 1987; Crabbe 1987; Baynes 1991; Raskin and Rosenstock 1992; Barnett 1993; Williamson et al. 1993; Wolff 1993; Giugliano et al. 1996).

Macrovascular disease, caused by atherosclerosis, is the major complication in diabetes accounting for 75% of all deaths (Bierman 1992). Several epidemiological studies have shown that the mortality rates from CHD are more than doubled in diabetic subjects compared with non-diabetic subjects (Garcia et al. 1974; Uusitupa et al. 1990a; Manson et al. 1991a; Stamler et al. 1993). The impact of CHD is greatest in type II diabetics, since approximately 80% of patients are in this category, therefore, the majority of deaths are seen in this group. However, it is clear that atherosclerosis is also increased in type I diabetes, even in the absence of proteinuria and nephropathy (Krolewski et al. 1987).

Environmental factors appear to be partly responsible for the increased prevalence of CHD, as exemplified by Japanese subjects resident in Hawaii. This group had more than double the rate of CHD, compared with similar diabetic subjects living in Japan, whilst the prevalence of microvascular complications were similar in both groups (Kawate et al. 1979).

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It appears that atherosclerosis occurs as a result of diabetes compounding other risk factors, such as hypertension and hyperlipidaemia. For example, the risk of atherosclerosis at any level of serum cholesterol, or in the presence of hypertension, smoking or obesity was 2-6 fold greater in diabetic subjects compared with non-diabetic subjects (Manson *et al*. 1991a; Stamler *et al*. 1993). However, even when all of the associated risk factors were taken into consideration, their presence could not account for the excessive increase in CHD, which was attributed to the diabetes *per se* (Garcia *et al*. 1974; Manson *et al*. 1991a; Stamler *et al*. 1993). Hence, other factors specific to diabetes appear to be involved, which result in accelerated atherosclerosis.

A multitude of factors can contribute to atherogenesis and it is unlikely that one factor can explain the increase in CHD found in diabetes. In recent years, attention has been turning to the role of free radical mediated damage of LDL, a molecule inherent in the formation of atherosclerotic lesions and the action of antioxidants in preventing this damage.

1.4.1 Non-enzymatic glycosylation and the formation of free radicals

One of the best characterized effects of excess blood glucose is the process of non-enzymatic glycosylation (the Maillard reaction). Glucose and other reducing sugars react with proteins by a non-enzymatic process called glycosylation (glucose only) or glycation (sugars in general). The carbonyl group of the sugar binds to a free amino group of a protein, forming a reversible adduct (Schiff base), which rearranges over time to form a more stable, but reversible, ketoamine (Amadori product, Figure 1.9). The Amadori products undergo an extensive range of reactions, including rearrangement, dehydration and fragmentation reactions, forming complex advanced glycation end products (AGEs), which remain irreversibly bound to proteins. On stable long-lived proteins, AGEs accumulate with time, forming inter- and intramolecular crosslinks and have been implicated in ageing (Lee and Cerami 1992; Monnier 1990). In diabetes, the process of glycosylation occurs at an accelerated rate and has been suggested as the common underlying biochemical basis leading to microvascular complications (Brownlee *et al*. 1988; Brownlee 1992; Brownlee 1994).

The dominant structures and pathways of AGE formation *in vivo* have been difficult to determine, since a compound may be generated by different routes, from different precursors. Three major pathways of the Maillard reaction, which lead to protein damage, have been identified by Monnier *et al.* (1993) and are summarized below:

1. Non-oxidative pathway

Reducing sugar → Glycation → Deoxyosones → Non-oxidative browning and crosslinking (e.g., pyrroles)

2. Glycoxidative pathway

Reducing sugar → Glycation $\xrightarrow{\text{O}_2 \text{ / fragmentation}}$ Glycoxidation (e.g., CML, pentosidine)

3. Autoxidative pathway

Reducing sugar $\xrightarrow{\text{O}_2 \text{ / metal ions}}$ Ketoaldehyde formation $\xrightarrow{\text{O}_2^{.-}, \text{H}_2\text{O}_2, \cdot\text{OH}}$ Lipid / protein oxidation, fragmentation and crosslinking

1. The Amadori products undergo **non-oxidative** reactions such as enolization reactions (Appendix 2), which can lead to the regeneration of the amine group, whilst the sugar undergoes dehydration forming 1, 3 or 4 deoxyosones, Figure 1.9 (Ledl 1990). Similar dehydration reactions of the Amadori compounds also lead to the formation of deoxyosones.

The deoxyosones are Maillard intermediates, which contain highly reactive dicarbonyl groups that undergo further cyclization / dehydration reactions forming a variety of AGEs. These include numerous heterocyclic ring compounds, such as pyrroles (e.g., pyrraline; Figure 1.9), which are formed from 3-deoxyosones and remain irreversibly bound to proteins (Ledl 1990). Pyrroles have been detected in serum albumin from diabetic subjects using immunological assays (Hayase *et al.* 1989).

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Protein
|
CH₂
|
NH₂

+

HC=O
|
HCOH
|
HOCH
|
HCOH
|
HCOH
|
CH₂OH

Glucose

⇌ -H₂O / +H₂O

Protein
|
CH₂
|
N
‖
CH
|
HCOH
|
HOCH
|
HCOH
|
HCOH
|
CH₂OH

Schiff base
(aldimine)

⇌

Protein
|
CH₂
|
NH
|
CH₂
|
C=O
|
HOCH
|
HCOH
|
HCOH
|
CH₂OH

Amadori product
(ketoamine)

+H₂O →

Protein
|
CH₂
|
NH₂

+

CH₃   HC=O   CH₂OH
| | |
C=O   C=O   C=O
| | ‖
C=O   CH₂   C=O
| | |
HCOH   HCOH   CH₂
| | |
HCOH   HCOH   HCOH
| | |
CH₂OH   CH₂OH   CH₂OH

} 1, 3 and 4 - deoxyosones

↓

HC=O   HC=O
| |
C=O   C=O
| |
CH   -H₂O ⇌ +H₂O   CH₂
‖ |
CH   CH₂
| |
HCOH   C=O
| |
CH₂OH   CH₂OH

+

NH₂
|
Protein
↓

CH₂OH—[pyrrole ring]—C=O / H
N
|
Protein

(Pyrrole, e.g., pyrraline)

Figure 1.9 *Formation of early non-enzymatic glycosylation products.*

2. Free radical reactions have been observed in the formation of AGEs. Baynes and colleagues found that Amadori products were oxidized in the presence of O_2 and transition metal ions, into carboxymethyllysine (CML) and a highly fluorescent compound called 'pentosidine' (Ahmed *et al.* 1986; Grandhee and Monnier 1991; Dyer *et al.* 1991). Carboxymethyllysine was formed by the cleavage of Amadori products in a pathway that involved ROS, whilst pentosidine was derived from the inter- and intramolecular crosslinking of arginine and lysine residues, Figure 1.10 (Sell and Monnier 1989). It was postulated that the formation of these AGEs involved free radical mediated reactions, judged by the fact that the reactions were inhibited in the absence of O_2 or by the addition of SOD, catalase, •OH-scavengers and metal ion chelators (Ahmed *et al.* 1986; Dyer *et al.* 1991).

Several other groups have also reported that Schiff bases / Amadori adducts are sources of $O_2^{•-}$, in the presence of metal ions, which can then lead to the formation of •OH, initiating further damage to surrounding molecules (Jones *et al.* 1987; Sakurai and Tsuchiya 1988; Mullarkey *et al.* 1990; Sakurai *et al.* 1990; Smith and Thornalley 1992; Taniguchi *et al.* 1994). The formation of AGEs, by the combination of glycation and oxidation reactions has been called **'glycoxidation'** and is outlined in Figure 1.9 (Baynes 1991).

Concentrations of CML and pentosidine were found to be elevated in patients with diabetes, compared with age-matched controls and correlated positively with the presence of complications (Monnier *et al.* 1986; Sell and Monnier 1990; Sell *et al.* 1993). These findings suggested that glycoxidation reactions occurred *in vivo* and could provide an indication of oxidative damage to proteins (Baynes 1991). However, the formation of CML and pentosidine does not appear to be limited to glucose, e.g., CML and pentosidine were formed during the reactions of proteins with the pentose-products of ascorbate oxidation shown in Figure 1.4 (Dunn *et al.* 1990; Nagaraj *et al.* 1991; Monnier *et al.* 1993); CML was also formed during lipid peroxidation reactions in the presence of protein (Fu *et al.* 1996). Thus, CML and pentosidine may be general indicators of oxidative damage in tissues (Fu *et al.* 1996).
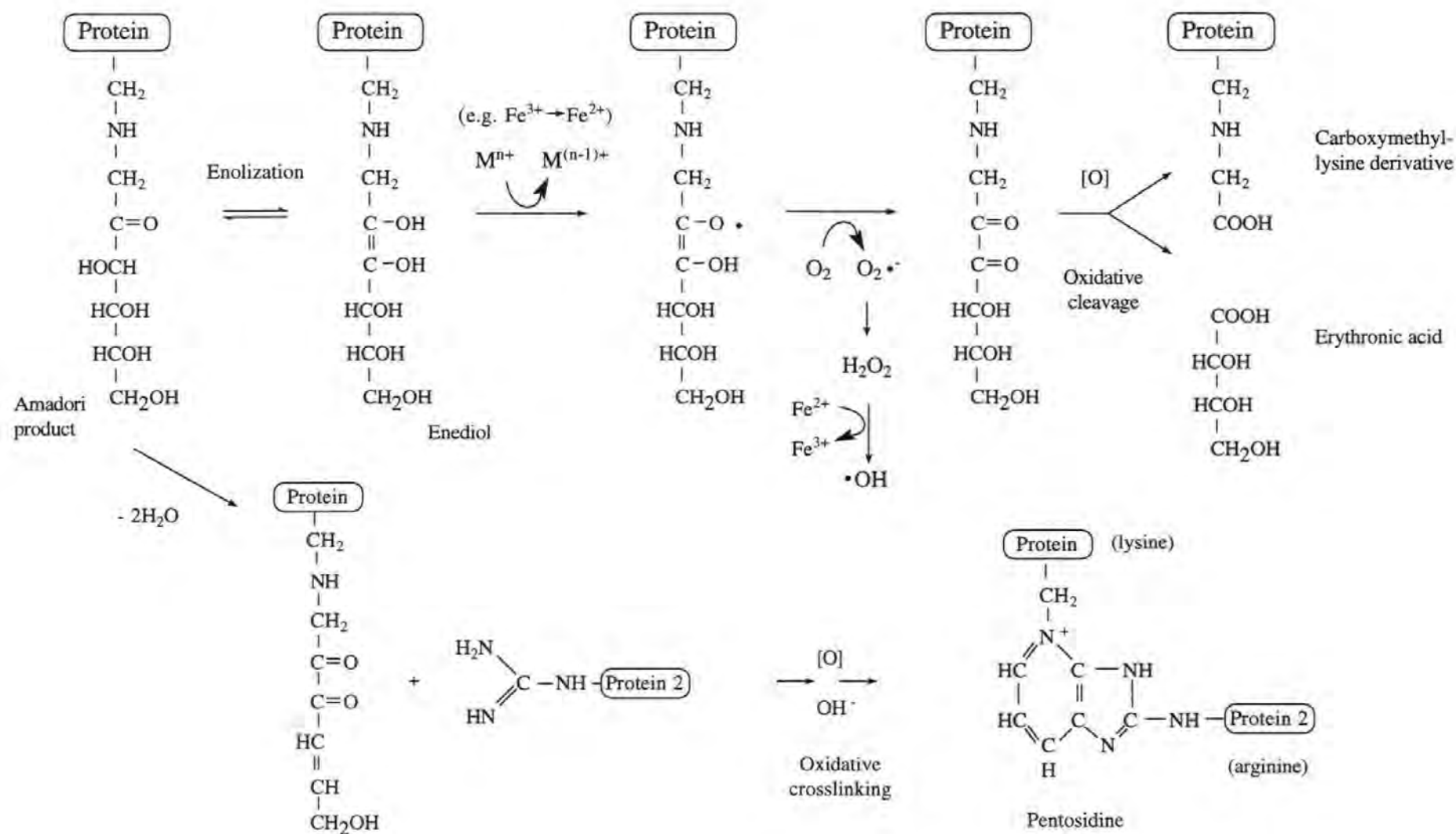
Figure 1.10 *Glycoxidation reactions of Amadori compounds (modified from Sakurai and Tsuchiya 1988). M^n indicates metal ions.*

Glycation of plasma proteins, including LDL apoproteins, is increased in diabetes (Lyons 1993). Since glycated proteins are sources of ROS, increased glycation of LDL might increase the oxidation of the lipid fraction and provide an explanation for the accelerated atherosclerosis found in diabetes (Mullarkey *et al*. 1990). Indeed, oxidation of LDL subsequent to glycation has been reported (Hunt *et al*. 1990; Sakurai *et al*. 1991; Bucala *et al*. 1993). Similarly, glycoxidative damage has been reported for SOD (Adachi *et al*. 1992; Ookawara *et al*. 1992; Taniguchi *et al*. 1994) and collagen (Hicks *et al*. 1988; Chace *et al*. 1991; Fu *et al*. 1992). Furthermore, glycosylation of intra- or extracellular SOD was associated with impaired activity of the enzyme (Arai *et al*. 1987; Adachi *et al*. 1992). Since levels of glycosylated SOD were found to be higher in diabetic subjects compared with controls, it has been suggested that this may also decrease antioxidant defences and contribute to vascular complications (Adachi *et al*. 1991).

3. In addition to glycosylation, glucose is known to undergo another type of reaction known as '**autoxidation**' (Wolff 1987; Wolff 1997). Glucose, in common with other α-hydroxy aldehydes, is able to enolize (Appendix 2) and reduce O_2 in the presence of transition metal ions, under physiological conditions of temperature and pH, yielding $O_2^{\cdot-}$, H_2O_2, $\cdot OH$ and reactive ketoaldehydes (Figure 1.11) (Wolff *et al*. 1984; Thornalley *et al*. 1984).

Evidence from *in vitro* studies suggests that the products of glucose autoxidation are a substantial cause of structural damage to proteins exposed to glucose (Wolff and Dean 1987; Hunt *et al*. 1988; Chace *et al*. 1991). Similar studies with LDL and erythrocyte membranes have shown that glucose initiates lipid peroxidation, by a pathway that involves $O_2^{\cdot-}$ (Hunt *et al*. 1990; Kawamura *et al*. 1994; Virgili *et al*. 1996). The ketoaldehyde products of glucose autoxidation were also found to react with proteins, contributing to glycosylation-related protein modification (Wolff and Dean 1987; Hunt *et al*. 1988).
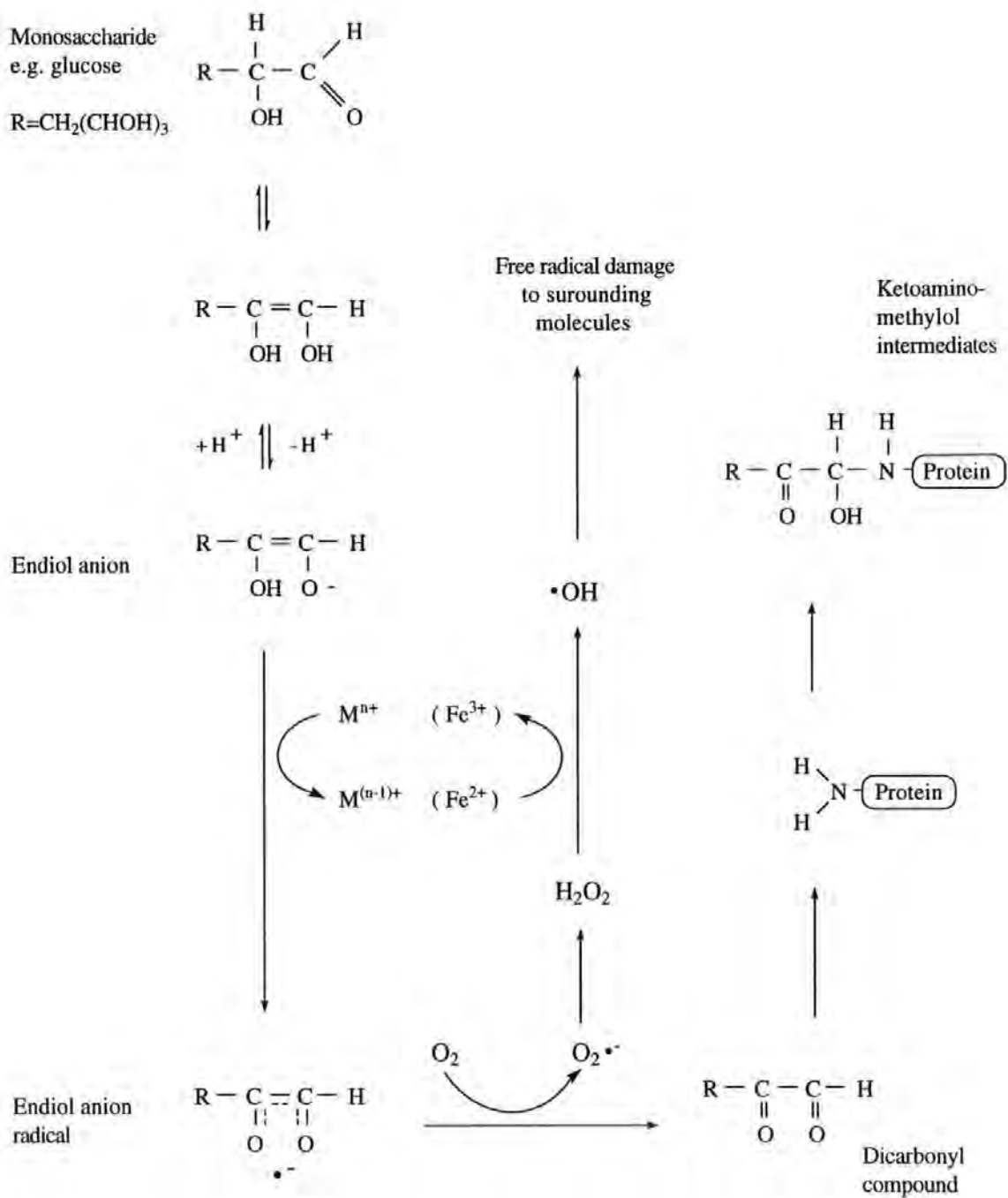
Figure 1.11 *Glucose autoxidation and the generation of free radicals (modified from Hunt et al. 1988; Wolff et al. 1991). M^n indicates metal ions.*

The reactivity of sugars had been known for sometime, with short chain sugars exhibiting greater reactivity than glucose, a reflection of the rate of ring opening (Bunn and Higgins 1981). It was Wolff's original suggestion that elevated concentrations of monosaccharides might be a source of reactive oxidants in diabetes (Wolff *et al*. 1984; Wolff 1987). Hence, hyperglycaemia may lead to an increase in the production of free radicals above normal basal levels, constituting a starting point for oxidative stress in diabetic patients (Gillery *et al*. 1989; Wolff 1993).

1.4.2   Oxidative stress and free radical activity in diabetes

Oxidative stress has been defined as an increase in the pro-oxidant / antioxidant balance, in favour of pro-oxidation, leading to potential damage (Sies 1991). Oxidative stress may occur due to an increase in the formation of endogenous free radicals which overwhelm antioxidant defences and / or inadequate antioxidant scavenging ability, enabling the number of free radicals to increase above normal basal levels, or both. A number of antioxidant defences appear to be compromised in diabetes and are discussed in the following section (1.5) and are shown in Appendix 3. It has been proposed that oxidative stress contributes to the pathogenesis of diabetic complications (Wolff 1987; Baynes 1991; Wolff *et al*. 1991; Wolff 1993; Giugliano *et al*. 1996). This hypothesis is based upon numerous studies, summarized in Appendix 3, which have reported elevated markers of free radical activity in persons with diabetes.

Sato *et al*. (1979) first reported increased levels of lipid peroxides in the plasma of diabetic subjects. This finding was subsequently confirmed by others who also measured lipid peroxides, mainly TBARS, as indirect indicators of free radical activity. In several studies, lipid peroxides were elevated in diabetic patients with complications, whilst those without complications had levels similar to those of the control groups (Sato *et al*. 1979; Jennings *et al*. 1987a; Jennings *et al*. 1991, Mooradian 1991). These results suggested that lipid peroxides were increased only in subjects with complications. Although, in some studies no mention of complications was made (Kaji *et al*. 1985; Gopaul *et al*. 1995). However, a

number of studies reported that lipid peroxides were significantly increased in diabetic patients compared with control subjects, regardless of the presence or absence of complications, supporting the view that diabetes is a condition pertaining to oxidative stress (Noberasco *et al.* 1991; Chittar *et al.* 1994; Gallou *et al* . 1994a; Parthiban *et al.* 1995; Sundaram *et al.* 1996). Elevated levels of lipid peroxides have also been reported in the erythrocyte membranes of diabetic subjects (Jain *et al.* 1989; Rajeswari *et al.* 1991; Parthiban *et al.* 1995).

Recent studies, using more specific markers of lipid peroxidation have added further support to the hypothesis that oxidative stress is increased in diabetes. A unique series of prostaglandin F_2-like compounds, known as 'isoprostanes', are formed during the autoxidation of arachidonic acid (Morrow *et al.* 1990; Morrow *et al.* 1992). The isolation and characterization of isoprostanes in plasma, by GC-MS, has provided a specific marker for lipid peroxidation *in vivo* (Morrow and Roberts 1996). Hence, the measurement of significantly elevated levels of isoprostanes in the plasma of type II diabetic patients has confirmed that lipid peroxidation is increased in diabetes (Gopaul *et al.* 1995). Similarly, another study using a 'precise' technique for measuring lipid peroxides, based upon the oxidation of ferric ions to ferrous ions, has also found elevated levels of lipid peroxides in diabetic subjects (Nourooz-Zadeh *et al.* 1995). Additionally, significantly greater amounts of oxidative damage to DNA were reported in the mononuclear cells of type I and type II diabetic patients compared with age-matched controls (Dandona *et al.* 1996). Furthermore, studies measuring the total peroxyl-radical trapping ability of the antioxidants (TRAP) in serum, have found significantly lower TRAP values in diabetic patients compared with control subjects, revealing that serum from diabetic subjects is more susceptible to lipid peroxidation (Asayama *et al* . 1993; Tsai *et al.* 1994; Ceriello *et al* . 1997). A further reduction in TRAP levels was also observed in patients with poor glycaemic control (Maxwell *et al.* 1997). These studies have all supported the view that oxidative stress is increased in diabetes.

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Changes in the patterns of antioxidant defences have also been reported in diabetic subjects. In general, a reduction in the activity of SOD was found in erythrocytes (Collier *et al.* 1990; Jennings *et al.* 1991; Sundaram *et al.* 1996) and leucocytes (Nath *et al.* 1984), indicating that there could be a possible increase in the formation of $O_2^{\cdot-}$. In poorly controlled diabetes, increased glycosylation of SOD may have been responsible for the decrease in enzyme activity (Adachi *et al.* 1991). An increase in the activity of erythrocyte GSH-Px has been observed, possibly as an adaptive mechanism against increasing oxidative stress (Kaji *et al.* 1985; Sundaram *et al.* 1996). Similarly, plasma caeruloplasmin levels were also significantly elevated in diabetic subjects (MacRury *et al*. 1993; Cunningham *et al.* 1995). Since caeruloplasmin functions as an 'acute-phase-reactant', elevated levels could be a further sign of high levels of oxidative stress.

Plasma levels of lipid peroxides were found to be significantly greater in diabetic subjects with vascular complications compared with subjects without complications (Armstrong *et al.* 1992; Collier *et al.* 1992; Gallou *et al.* 1993; Chittar *et al*. 1994; Gallou *et al.* 1994a; Parthiban *et al.* 1995; Sundaram *et al.* 1996). A progressive increase in plasma TBARS was also observed with increasing duration of diabetes and with increasing number of secondary complications (Armstrong *et al.* 1992; Sundaram *et al.* 1996). Interestingly, diabetic patients without vascular complications, but with hypertension, also had higher plasma TBARS than normotensive patients; whereas in patients with vascular disease, there were no differences between hypertensive and normotensive patients (Gallou *et al*. 1994a). One study has found significantly greater levels of TBARS in diabetic patients with microvascular complications, but with no evidence of macrovascular disease, compared with non-diabetic subjects with macrovascular disease (Belch *et al.* 1995). From these results it was postulated that the increased degree of oxidative stress in patients with microangiopathy may promote the development of atherosclerosis. Furthermore, patients with poor glycaemic control were also found to have higher levels of lipid peroxides than patients with good glycaemic control, in plasma (Noberasco *et al.* 1991; Armstrong *et al.* 1992; Griesmacher *et al.* 1995; Sundaram *et al.* 1996) and in erythrocytes (Jain *et al.* 1989; Sundaram *et al.* 1996).

It appears that diabetes is a condition associated with increased lipid peroxidation, particularly in patients with complications or poor glycaemic control. Plasma lipid concentrations may be important determinants of lipid peroxide levels and several studies found significant positive correlations between TBARS and cholesterol and triglyceride concentrations, indicating the possible importance of substrate availability (Velázquez *et al.* 1991; Gallou *et al.* 1994a; Griesmacher *et al.* 1995). Others, however, found no correlations between TBARS and glycaemic control and plasma lipid levels, suggesting that plasma lipid peroxide levels were not simply a function of plasma lipid concentrations or a consequence of hyperglycaemia (Gopaul *et al.* 1995; Nourooz-Zadeh *et al.* 1995).

Interpretation of the data from studies of lipid peroxidation in diabetes have been confounded by reports that plasma lipid peroxides are also increased in non-diabetic subjects with vascular disease (Stringer *et al.* 1989; Velázquez *et al.* 1991; MacRury *et al.* 1993; Belch *et al.* 1995) or hyperlipidaemia (Chirico *et al.* 1993; Nacitarhan *et al.* 1995). Hence, the increase in plasma lipid peroxides in diabetic patients may simply be an indication of the background level of atherosclerosis, which has not yet manifested the clinical symptoms of the disease. In this way, the TBAR test has been put forward as a diagnostic tool for the early detection of patients at risk from cardiovascular events (Gallou *et al.* 1994a; Griesmacher *et al.* 1995).

Despite the numerous studies confirming the presence of oxidative stress in diabetes, the role and origin of oxidative stress is less clear. It is not known whether increased lipid peroxides are the cause or the result of the long term complications and the source(s) of free radicals has not been established. Interpretation of the role of oxidative stress in the pathogenesis of diabetic complications has been made difficult by the fact that once tissue damage has occurred oxidative stress may then continue in a self-perpetuating 'vicious circle' (Baynes 1991). However, several studies have indicated that changes occur at an early stage in diabetes and possibly before the development of complications (Asayama *et al.* 1993; Parthiban *et al.* 1995; Sundaram *et al.* 1996).

In contrast, studies using animals as a models for diabetes have clearly shown a free radical involvement in the pathogenesis of diabetes and its complications. The pancreatic ß-cells are poorly equipped in terms of the antioxidant enzymes, SOD and GSH-Px (Grankvist *et al.* 1981a; Malaisse *et al.* 1982), and are destroyed by autoimmune processes mediated by cytokines and free radicals (Oberley 1988; Rabinovitch 1992; Nerup *et al.* 1994). Consequently, the non-obese diabetic (NOD) mouse and the Biobreeding (BB) rat develop type I diabetes after the release of ROS, which occurs during macrophage and T-cell lymphocyte infiltration of the pancreatic islets (Horio *et al.* 1994; Brenner *et al.* 1993). Similarly, the diabetogenic drugs streptozotocin (STZ) and alloxan selectively destroy the pancreatic ß-cells by free radical mediated processes (Uchigata *et al.* 1982). Administration of antioxidants prior to the development of diabetes, prevented or reduced the incidence of damage to the pancreatic islets, supporting the concept of a free radical involvement (Grankvist *et al.* 1981b; Uchigata *et al.* 1982; Horio *et al.* 1994). After the onset of diabetes, levels of lipid peroxides were found to increase in the plasma and tissues of diabetic rats (Suresh Kumar and Menon 1992; Young *et al.* 1992) and were corrected by insulin treatment or lessened by antioxidant therapy (Morel and Chisolm 1989; Young *et al.* 1992). Furthermore, reports that antioxidants restored nerve function in diabetic rats have implied a role for ROS in the aetiology of the complications (Cameron *et al.* 1994; Cameron and Cotter 1995; Cotter *et al.* 1995).

In summary, diabetes mellitus is associated with a high prevalence of vascular complications, with cardiovascular disease as the leading cause of mortality in the majority of patients. Hyperglycaemia has been established as the leading cause of the vascular complications (DCCT 1993), but the mechanisms by which hyperglycaemia leads to vascular disease are not fully understood. Numerous studies have reported elevated levels of lipid peroxides in diabetic patients and oxidative stress is thought to be a contributing factor to the development of complications, particularly atherosclerosis (Giugliano *et al.* 1996). The hypothesis that oxidative stress contributes to the development of diabetic complications is an attractive one, because the consequences of hyperglycaemia may be modulated by the ability of individuals to withstand oxidative stress and may explain the

individual variation in susceptibility to complications (Lyons and Johnson 1994).

The glycoxidation and autoxidation pathways are potential sources of free radicals *in vivo*, which may potentiate protein damage by increasing crosslinking, fragmentation and the formation of AGEs (Hunt and Wolff 1991; Hunt *et al*. 1993). Since proteins are found in close proximity to lipids, the generation of reactive molecules can potentially initiate the autocatalytic reactions of lipid peroxidation. Hence, an increase in the production of free radicals by these mechanisms has been linked to the development of oxidative stress, microvascular complications and atherogenesis in diabetes, and also the ageing process (Wolff 1987; Baynes 1991; Wolff *et al*. 1991; Hunt *et al*. 1990; Mullarkey *et al*. 1990; Kristal and Yu 1992).

Numerous *in vitro* studies have shown that the autoxidation and glycoxidation reactions of glucose are sources of ROS, but clear evidence that these reactions occur *in vivo* and may be responsible for the elevated levels of lipid peroxides found in diabetic patients is lacking. Thus, the effect of acute episodes of hyperglycaemia on lipid peroxidation and antioxidant vitamin levels were investigated as part of this study.

1.5 Antioxidant Vitamin Status in Diabetes

In type I and type II diabetes, alterations in trace element and antioxidant vitamin status are found (reviewed by Strain 1992; Mooradian *et al.* 1994; Thompson and Godin 1995).

Vitamin E

Studies with experimental animals have found that plasma and tissue levels of vitamin E are elevated after the onset of diabetes (Behrens *et al*. 1984; Pritchard *et al*. 1986). In clinical studies, plasma levels of vitamin E were elevated in both type I and type II diabetic subjects, when compared with control subjects (Vatassery *et al*. 1983; Caye-Vaugien *et al.* 1990; Krempf *et al.* 1991) and showed an increase with age (Lewis *et al.* 1973; Martinoli *et al.* 1993). However, as plasma lipids tend to be elevated in diabetes, lipid standardized vitamin E levels were calculated and found to be normal (Vandewoude *et al.* 1987; Martinoli *et al.* 1993). Several studies have indicated that vitamin E status is deficient in the platelets of diabetic persons and may enable an increase in the production of thromboxane A2 and increased platelet aggregation (Watanabe 1984; Karpen 1985; Gisinger *et al.* 1990).

Dietary supplementation with vitamin E may reduce protein glycation in diabetes. Ceriello *et al.* (1991a) observed a significant reduction in glycosylated haemoglobin and in the glycosylation of other proteins in type I diabetic subjects, after 2 months of supplementation with either 600 or 1200 mg of vitamin E per day; the response was also dose related. Modest improvements in glycaemic control, due to increased insulin sensitivity, were also reported with supplements of 900 mg daily for 4 months in type II diabetic subjects (Paolisso *et al.* 1993) and in elderly non-diabetic subjects (Paolisso *et al*. 1994). In contrast, Reaven *et al*. (1995) found no evidence of improvements in fasting serum glucose or protein glycation in type II diabetic patients given 1600 IU of vitamin E daily for 10 weeks, but a significant increase in LDL vitamin E levels and resistance of LDL to oxidation was observed. Parfitt *et al.* (1996) found no change in glycaemic control in type I diabetic patients consuming moderate supplements (400 IU (269 mg)) of vitamin E per day for 8 weeks, nor any reduction in lipid peroxidation; although, a subgroup of patients did show a significant enrichment in LDL vitamin E and a subsequent reduction in lipid peroxidation.

Reaven *et al.* (1995), suggested that the ability of vitamin E to inhibit protein glycation might have been limited to those subjects who had poor diabetic control and higher rates of protein glycation. Additionally, vitamin C or a combination of antioxidants might have been responsible for the effects on glycation (Dunn *et al.* 1990; Shoff *et al.* 1993; Reaven *et al.* 1995; Vinson and Howard 1996). However, the duration of supplementation is a likely factor, since other studies with longer periods of supplementation have found a reduction in glycosylated haemoglobin (Duntas *et al.* 1996; Jain *et al.* 1996a).

Antioxidant supplementation increases the resistance of LDL to oxidation in healthy individuals (Dieber-Rotheneder *et al.* 1991; Jialal and Grundy 1992; Reaven *et al.* 1993a). Vitamin E supplements were found to produce similar effects in type I and II diabetic subjects (Reaven *et al.* 1995; Fuller *et al.* 1996). Supplementation with vitamin E was also found to decrease platelet aggregation (Colette *et al.* 1988; Gisinger *et al.* 1988) and plasma lipid peroxide levels in diabetic patients (Jain *et al.* 1996b), thereby providing further anti-atherogenic protection. Vitamin E supplementation offers potential benefits in the treatment of diabetes, but the long term effects of supplementation are unknown at present and cannot be recommended in clinical practice (Garg 1996; Gazis *et al.* 1997).

Vitamin A

Serum retinol and RBP concentrations were found to be significantly decreased in children and adults with type I diabetes, when compared with non-diabetic control subjects (Basu *et al.* 1989; Krempf *et al.* 1991; Martinoli *et al.* 1993). In an earlier study, in type I diabetic subjects, reduced plasma retinol concentrations were found, whilst the levels of retinyl esters associated with lipoproteins were significantly elevated (Wako *et al.* 1986). The cause of the reduced plasma levels of retinol in diabetes is uncertain. It has been suggested that hepatic storage of retinol is increased, whilst the release of retinol by the liver and transport in plasma is affected in diabetes (Basu *et al.* 1989). This theory was supported by Basu *et al.* (1990) and Tuitoek *et al.* (1996), who reported that plasma levels of retinol and RBP were significantly reduced in rats made diabetic with STZ, whilst hepatic stores of the vitamin gradually increased after the onset of diabetes.

In contrast, a significant increase in plasma retinol concentrations was reported in type II diabetic subjects, compared with age-matched controls (Krempf *et al*. 1991). In a recent study, no differences in plasma retinol concentrations were found between type II diabetic subjects and control subjects; however, the levels of RBP were significantly higher in the diabetic group and highest in those patients receiving insulin therapy (Basualdo *et al*. 1997).

Plasma retinol concentrations are regulated by the synthesis and release of RBP by the liver, a process that requires zinc and protein (Smith 1980; Olson 1991). Zinc status is altered in diabetes (reviewed by Strain 1992; Thompson and Godin 1995) and insulin is also known to affect vitamin A metabolism and *vice versa* (Bowles 1967; Chertow *et al*. 1987; Chertow *et al*. 1993). Thus, several factors are likely to be involved in altering the plasma retinol concentrations, as a direct consequence of the diabetic state. It has been suggested that in type I diabetes, insulin deficiency may impair the release of retinol by the liver, causing a reduction in the plasma retinol concentrations, whereas hyperinsulinaemia may be responsible for the elevation in retinol and RBP observed in type II diabetes (Krempf *et al*. 1991; Basualdo *et al*. 1997).

There is a lack of data available on the effects of vitamin A supplementation in diabetic patients. However, the majority of type II diabetic patients are unlikely to be deficient in vitamin A and large doses are known to have toxic effects; thus, the use of vitamin supplements are not justified in diabetes (Mooradian *et al*. 1994; Garg 1996). Indeed, two recent studies have highlighted the need for caution when proceeding with antioxidant supplementation therapy: The Alpha Tocopherol Beta Carotene Cancer Prevention Study (ATBC (1994)), and the Beta Carotene and Retinol Efficacy Trial (CARET (Omenn *et al*. 1996)), were originally designed to test for the possible effects of vitamin E, ß-carotene and retinol supplementation, on the prevention of lung cancer in high risk groups. In the ATBC study, male smokers were randomized to vitamin E (50 mg per day), ß-carotene (20 mg per day), vitamin E plus ß-carotene or placebo. In the CARET study, patients received either a combination of 30 mg of ß-carotene and 25000 IU of retinol per day, or placebo. Both studies showed an excessive increase in the incidence of lung cancer in those treated with

supplements and no evidence of a beneficial effect of supplementation. As a result, the CARET intervention was stopped prematurely. These somewhat alarming results, emphasized the need for a greater understanding of the physiological functions of antioxidants and their interactions, in both healthy and diseased states, before considering the use of antioxidant therapy.

Vitamin C

Ascorbic acid status is disrupted in diabetes mellitus (Will and Byers 1996). In animals with experimentally induced diabetes, decreases in plasma and tissue AA concentrations have been observed (Yew 1983; Schlosser *et al*. 1987; McLennan *et al*. 1988; Yue *et al*. 1989; Yue *et al.* 1990). Alterations in AA status were also found in the diabetes prone BB rat before the onset of the disease (Behrens and Madere 1991).

Numerous studies have indicated that in both type I and type II diabetic subjects, plasma levels of AA are significantly lower than those of non-diabetic controls (Chaterjee and Banerjee 1979; Som *et al*. 1981; Jennings *et al*. 1987b; Yue *et al*. 1990; Sinclair *et al.* 1991; Sinclair *et al*. 1994; Seghieri *et al.* 1994). In addition, diabetic subjects with complications were found to have significantly lower plasma ascorbate levels than those without complications (Sinclair *et al*. 1991). Similarly, levels of AA were found to be reduced in white cells and platelets, suggesting that tissue storage of AA was also impaired in diabetes (Chen *et al.* 1983; Cunningham *et al*. 1991). However, the findings are not consistent; in type I and II diabetic subjects with high dietary intakes of vitamin C, plasma and white cell ascorbate concentrations were found to be similar to those of the control groups (Stankova *et al*. 1984; Schorah *et al*. 1988; Lysy and Zimmerman 1992).

Since plasma levels of AA reflect intake, low plasma levels may be indicative of an inadequate dietary supply of the vitamin. When this factor was investigated, no significant differences were found between the dietary intakes of diabetic and non-diabetic subjects, but the plasma concentrations of AA were significantly lower in the diabetic group (Sinclair *et al.* 1994). This finding added further support to the theory that disturbances in AA metabolism

occurred as a result of the diabetes.

The exact mechanisms to account for the lowering of AA are uncertain. It has been suggested that AA and glucose (and also DHAA), share a common membrane transport mechanism, due to the structural similarity between the molecules (Mann and Newton 1975; Bigley et al. 1983; Davis et al. 1983). Thus, competition between glucose and AA for the carrier may be responsible for the lowering of AA concentrations in plasma and tissues. This theory was supported by in vitro studies which showed that the uptake of AA by lymphocytes, and DHAA by leucocytes and fibroblasts was inhibited by glucose (Bigley et al. 1983; Davis et al. 1983; Stankova et al. 1984; Cunningham 1988). The effect of acute hyperglycaemia in vivo, in healthy subjects, also caused an acute decrease in the concentration of AA in leucocytes, suggesting that plasma glucose may be an important factor inducing the intracellular depletion of AA (Chen et al. 1983; Pecoraro and Chen 1987).

Yue et al. (1990) and Lysy and Zimmerman (1992) found a negative correlation between plasma AA concentrations and glycosylated haemoglobin and reported that tissue depletion of AA did occur with increasing hyperglycaemia. However, Som et al. (1981), Jennings et al. (1987b), and Sinclair et al. (1991) found no relationship between AA concentrations and glycaemic control and concluded that AA levels were not lowered as a direct consequence of hyperglycaemia. The in vitro studies have shown that different cell types take up AA and DHAA at different rates (Mooradian 1987), suggesting that there may be at least two transport mechanisms operating (reviewed by Cunningham 1988; Schorah 1992; Levine et al. 1994), one of which is impaired by hyperglycaemia. In cells where the accumulation of AA occurs by the uptake of DHAA, followed by reduction to AA, insufficient concentrations of cellular reducing agents could impair the uptake of DHAA (Schorah 1992).

The reduction of DHAA back to AA, to maintain its antioxidant activity, is coupled with GSH and glutathione reductase activity, which in turn is dependent upon the production of NADPH by the hexose monophosphate shunt pathway (and glucose 6-phosphate dehydrogenase activity). In cells where the entry of glucose is not affected by insulin,

notably the lens, retina, erythrocytes, glomerulus and peripheral nerve the polyol pathway (Figure 1.3, page 18) may be responsible for lowering NADPH levels, increasing the NADH/NAD+ ratio (Williamson *et al.* 1993) and, hence, decreasing the cellular reducing capacity necessary for the reduction of DHAA in cells. Indeed, low concentrations of GSH have been reported in diabetes (Murakami *et al.* 1989; Costagliola 1990). Similarly in muscle, adipose tissue and liver where insufficiencies in the action of insulin result in decreased intracellular glucose concentrations, the activity of the hexose monophosphate shunt pathway is impaired, resulting in decreased NADPH and GSH levels. Thus, changes in cellular redox potentials may underlie the alterations in AA metabolism, since numerous cellular processes, including the reduction of DHAA to AA, cellular uptake of DHAA and the regeneration of other antioxidants are likely to be affected.

Urinary excretion of AA was found to correlate negatively with glycosylated haemoglobin, suggesting that excessive urinary losses did not occur in diabetic patients with poor glycaemic control (Yue *et al.* 1990). Seghieri *et al.* (1994) found that renal clearance of AA was altered in type I diabetes, but also concluded that urinary losses did not account for the reduced serum levels that were observed.

Ascorbic acid also functions as an important water-soluble antioxidant in plasma (Frei *et al.* 1989). A decrease in the concentration of AA, or an increase in the ratio of DHAA/AA, may be indicative of an increase in the consumption of AA due to oxidative stress. Dehydroascorbic acid levels in diabetic subjects have been reported to be significantly higher (Chaterjee and Banerjee 1979; Som *et al.* 1981; Banerjee *et al.* 1982), significantly lower (Sinclair *et al.* 1994) and similar (Newill *et al.* 1984; Stankova *et al.* 1984; Sinclair *et al.* 1991) to those found in control subjects. These discrepancies may reflect methodological difficulties in assessing the levels of DHAA. Indeed, there is a great deal of variation in the published values of DHAA in non-diabetic subjects as well as in diabetic subjects, as indicated in Table 1.6. Despite the analytical difficulties in measuring DHAA, the ratio of DHAA/AA was found to be greater in diabetic patients compared with control subjects (Jennings *et al.* 1987b; Sinclair *et al.* 1991).

Table 1.6 *Plasma levels of ascorbic and dehydroascorbic acid in diabetic and non-diabetic subjects.*

Reference	Type of Diabetes	Diabetic subjects		Control subjects	
		AA	DHAA	AA	DHAA [a]
Sinclair *et al.* 1994	I & II [b]	30.4 ± 19.1 (n = 20)	27.6 ± 6.4	68.8 ± 36.0 (n = 20)	31.8 ± 4.8
Sinclair *et al.* 1991	II	55.6 ± 20 42.1 ± 19.3 (n = 20)	30.5 ± 10.8 [c] 31.3 ± 9.9 [d]	82.9 ± 30.9 (n = 22)	28.8 ± 12.6
Jennings *et al.* 1987b	I & II	42.5 ± 26.2 (n = 38)	18.8 ± 7	58 ± 21 (n = 20)	19.2 ± 6.9
Newill *et al.* 1984	I & II	32 ± 14 (n = 17)	5 ± 8	47 ± 26 (n = 12)	7 ± 5
Banerjee 1982	II	17.04 ± 17 (n = 57)	40.9 ± 8.5	48.85 ± 9.1 (n = 96)	1.7 ± 2.3
Chaterjee & Banerjee 1979	II	9.6 ± 6.2 (n = 12)	12.6 ± 2.3	21.6 ± 15.3	0
Ødum 1993				58 ± 14 (n = 10)	2.7 ± 2.5
Nagy and Degrell 1989				23.2 ± 17.3 (n = 10)	5.8 ± 2.7
Lunec and Blake 1985				57.7 ± 19.6 (n = 20)	12.0 ± 3.7
Okamura 1980				55.1 ± 6.6 (n = 10)	1.44 ± 1.7

[a] Plasma ascorbic (AA) and dehydroascorbic acid (DHAA) concentrations (μmol/l) expressed as mean ± SD.

[b] I = Type I and II = Type II diabetes mellitus.

[c] Diabetic patients without microangiopathy.

[d] Diabetic patients with microangiopathy.

The ratio of DHAA/AA was also significantly higher in diabetic patients with microangiopathy compared with diabetic patients without complications, suggesting that oxidative stress was greater in diabetic patients with complications (Sinclair *et al.* 1991).

Alterations in AA status may affect cholesterol metabolism in diabetes, since AA is involved in the activity of cholesterol-7-α-hydroxylase, the rate limiting enzyme regulating the conversion of cholesterol to bile acids (Basu and Dickersen 1996). Ascorbate deficient guinea-pigs developed hypercholesterolaemia as a result of the impaired activity of the enzyme (Ginter and Bobek 1981). In man, epidemiological studies have indicated that vitamin C is inversely associated with serum cholesterol and correlates positively with high-density lipoprotein (HDL) cholesterol (Jacques *et al.* 1987; Simon 1992; Jacques *et al.* 1994; Hallfrisch *et al.* 1994); although the data are not consistent, probably as a result of the initial AA status and other environmental factors. Supplementation with AA was found to produce a cholesterol-lowering effect in type II diabetic and non-diabetic people whose initial AA status was low (Ginter and Bobek 1981), and to elevate HDL cholesterol (Horsey *et al.* 1981). However, there is no evidence that increased AA intake, above requirements, will result in an increase in cholesterol catabolism, which is perhaps why other intervention studies have shown no effect on total cholesterol levels (Peterson *et al.* 1975; Khan and Seedarnee 1981; Bishop *et al.* 1985).

Ascorbic acid is an essential cofactor in the biosynthesis of collagen. Hence, AA deficiency has been implicated in collagen abnormalities leading to the long term complications seen in diabetes (McLennan *et al.* 1988). Supplementation with ascorbate was found to reduce vascular fragility (Cox and Butterfield 1975); sorbitol accumulation in erythrocytes, both *in vitro* and *in vivo* (Vinson *et al.* 1989; Cunningham 1994); decrease the glycation of proteins and haemoglobin *in vivo* (Davie *et al.* 1992) and reduce blood pressure (Ceriello *et al.* 1991b). Partial restoration of plasma AA levels was also achieved with supplementation (Som *et al.* 1981; Sinclair *et al.* 1991). However, upon the discontinuation of supplements, plasma AA levels were found to decrease rapidly in the diabetic subjects compared to the control group (Som *et al.* 1981).

Supplementation appears to be of benefit in diabetes, but pro-oxidant properties of AA were observed in animal studies (Chen 1981; Young *et al.* 1992), and megadoses (2 g per day) were found to delay the insulin response to a glucose challenge in healthy subjects (Johnston and Yen 1994). These studies have emphasized the need for caution, when proceeding with the use of large supplements for diabetic patients.

Uric acid

Interesting relationships have been reported between serum uric acid and glucose concentrations in diabetic subjects (Cook *et al.* 1986; Olukoga *et al.* 1991; Whitehead *et al.* 1992). Prediabetic subjects were found to have higher serum uric acid levels than non-diabetic subjects and diabetic patients were found to have lower uric acid levels than non-diabetic subjects (Herman and Goldbourt 1982; Cook *et al.* 1986). An increase in serum urate was observed with increasing plasma glucose concentrations up to 8 mmol/l. Thereafter, as glucose concentrations increased urate concentrations decreased (Cook *et al.* 1986; Whitehead *et al.* 1992). The low serum urate levels appeared to result from increased urinary loss induced by the chronic hyperglycaemia and glycosuria (Cook *et al.* 1986; Olukoga *et al.* 1991). This fall in urate concentrations may further weaken antioxidant defences in diabetic patients with increasing hyperglycaemia (Whitehead *et al.* 1992).

1.6 Obesity and Type II Diabetes

Obesity is characterized by an excess of body fat and gain in body weight. The body mass index (BMI), calculated as weight (kg) / height (m^2), is the measure most commonly used to classify obesity. Tables of weight relative to height, prepared by The Metropolitan Life Insurance Company, have also been used to assess the degree of overweight (Garrow 1988). The categories of BMI for adults are:

Ungraded :	BMI < 20	Underweight
Grade 0 :	BMI 20-24.9	Normal / desirable weight
Grade 1 :	BMI 25-29.9	Overweight
Grade 2 :	BMI 30-40	Obese
Grade 3 :	BMI > 40	Severely obese

The prevalence of obesity is increasing at an alarming rate worldwide (Van Itallie 1994). In the UK, the prevalence of obesity increased from 6% to 13% in men and from 8% to 15% in women, between 1980 and 1991 (White *et al.* 1993). In 1991, the Government launched *The Health of the Nation* initiative, one of the aims of which was to reduce the prevalence of obesity back to the 1980 levels by the year 2005 (Secretary of State for Health 1991). Later surveys have shown that the prevalence of obesity has continued to rise, with 13% of men and 16% of women classifying as obese in 1993 (Bennett *et al.* 1995). If the trends persist, it is estimated that 18% of men and 24% of women will be obese by 2005 (Garrow 1996).

Obesity develops when there is a sustained increase in energy intake, in excess of energy expenditure. Although there is a genetic component in the development of obesity, this accounts for approximately 25% of the variability between individuals (Livingstone 1996). A multitude of other factors, such as socio-economic, psychological and cultural can contribute to the development of obesity. However, the interaction between physical inactivity and increased fat consumption relative to carbohydrate intake, are probably the key environmental factors responsible for the alarming increase in obesity (Prentice and Jebb 1995). Other studies have also shown that physical inactivity is an important determinant in the development of obesity and type II diabetes (Schulz and Schoeller 1994; Rising *et al.* 1994; Manson *et al.* 1991b; Helmrich *et al.* 1991; Manson *et al.* 1992).

Overweight and obesity are well known risk factors for disease, including dyslipidaemias, gall bladder disease, respiratory disease, certain cancers, hypertension and osteoarthritis (Garrow 1991; Kanders *et al*. 1994). The incidence of CHD is high in obese people (Hubert *et al*. 1983; Jarrett *et al*. 1982; Manson *et al*. 1990) and there is also a strong positive association between BMI and the development of type II diabetes (Perry *et al*. 1995). Hence, it is estimated that 60-90% of type II diabetic patients in Western countries are obese (Harris and Zimmet 1992).

In addition to the BMI, the distribution of fat has important implications on the development of CHD. People with abdominal (central) obesity are at greater risk of death from CHD than those in which the distribution of fat is more peripheral. It is now recognized that abdominal obesity (indicated by a high waist / hip ratio), is a stronger predictor of total mortality and death from CHD than overall obesity, i.e., BMI (Larsson *et al*. 1984; Lapidus *et al*. 1984; Bengtsson *et al*. 1993). Furthermore, the majority of type II diabetic patients present with characteristic abdominal obesity, a feature first recognized by Vague in the 1940s (Vague 1956). Epidemiological studies subsequently confirmed that individuals with abdominal obesity have a high risk of developing type II diabetes (Kalkhoff *et al*. 1983; Ohlsen *et al*. 1985).

1.6.1   The insulin resistance syndrome

Abdominal obesity is associated with metabolic aberrations (Kissebah 1982), including dyslipidaemia (increased very low density lipoproteins (VLDL) and reduced HDL levels), impaired glucose tolerance (IGT), hypertension and insulin resistance. These factors have been grouped together and were defined as the metabolic syndrome (syndrome X) or the insulin resistance syndrome, by Reaven and Olefsky in the 1980s (Olefsky 1982; Reaven 1988; DeFronzo and Ferrannini 1991). The syndrome is characterized by three main features: visceral accumulation of fat, accompanied by metabolic derangements (in fat and carbohydrate metabolism, of which insulin resistance is a key feature) and an endocrine and central nervous system aberration (Björntorp 1992).

Adipocytes in the abdominal region appear to be uniquely equipped in terms of the density of hormone receptors, blood flow and nervous innervation, forming a region of adipose tissue with high metabolic activity. Enlarged visceral adipocytes have a high turnover of lipids, with increased flux of free fatty acids (FFA) into the portal vein. This leads to elevated gluconeogenesis, with the risk of hyperglycaemia and stimulates an increase in the production of triglycerides by the liver, leading to elevated VLDL and LDL concentrations. As FFA levels rise, the response of the liver to insulin is decreased and the hepatic clearance of insulin is reduced, increasing systemic insulin levels. Insulin-mediated glucose uptake and utilization by muscle is also suppressed as systemic concentrations of FFA increase, contributing to peripheral insulin resistance (Randle et al. 1963; Björntorp 1992).

In order to compensate for these and other factors, the requirement for insulin is increased, and a state of 'insulin resistance' is induced, whereby higher insulin levels are required to metabolize a given glucose load (i.e., a normal amount of insulin would produce an subnormal biological response) (Reaven 1988; Beck-Nielsen 1992; Björntorp 1992). The increased secretion of insulin produces a state of hyperinsulinaemia, defined by elevated systemic insulin levels. This can itself lead to insulin resistance, by down regulating insulin receptors, thereby perpetuating the insulin resistance syndrome in a 'vicious circle'. Thus once established, the exact mechanisms which produce insulin resistance are difficult to determine (Björntorp 1992). Peripheral obesity may also be associated with insulin resistance, but to a lesser extent than abdominal obesity, due to increased insulin secretion and less efficient hepatic clearance, as well as a reduced response of target cells to insulin (Björntorp 1992). Hyperinsulinaemia also stimulates the sympathetic nervous system (SNS), in what has been hypothesized as a physiological adaptation to obesity. The increase in SNS activity increases thermogenesis, preventing further weight gain in obese subjects, by restoring energy balance (Daly and Landsberg 1991).

The insulin resistance syndrome also encompasses a further endocrine component. Energy balance is influenced in the short term by a number of hormones, including thyroid hormone, growth hormone, glucocorticoids and adrenaline. The endocrine abnormalities

associated with abdominal obesity include disturbances in sex steroid hormones and increased levels of glucocorticoids (reviewed by Björntorp 1992). Adipocytes in the abdominal region also appear more sensitive, than peripheral fats cells, to the action of catecholamines and cortisol. The high lipolytic activity of abdominal fat cells, in response to hormone levels, e.g., during stress, can expose the liver to high levels of FFA. Hence, endocrine disturbances affect lipolysis, modify fat distribution and induce insulin resistance (Björntorp 1992). Other hormones which may have a role in insulin resistance include leptin, the product of the obesity (ob) gene (Zimmet et al. 1997).

The primary consequence of abdominal obesity appears to be reduced sensitivity to insulin, from which other metabolic disturbances follow. Obesity is, therefore, a state of compensated insulin resistance, wherein hyperinsulinaemia counteracts the pathways that increase the requirement for insulin, in order to maintain normal plasma glucose concentrations. However, if the pancreatic ß-cells are unable to produce enough insulin to compensate for insulin resistance, then hyperglycaemia increases, resulting in IGT (fasting plasma glucose (FPG) 6.1 to < 7.0 mmol/l) and then frank type II diabetes (FPG ≥ 7.0 mmol/l) (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997). Type II diabetes, therefore, develops as a result of impaired insulin action and insulin secretion, caused by a combination of genetic and environmental factors (Sacks and McDonald 1996). Furthermore, different ethnic groups appear to be more susceptible to the insulin resistance syndrome. For example, the prevalence of diabetes was found to be greater in South Asians resident in the UK, compared with the European population. This group showed a striking tendency to central obesity, with the associated metabolic disturbances, resulting in higher mortality rates than the European group (McKeigue et al. 1991). Other ethnic groups susceptible to insulin resistance, include the Australian Aborigines and the Pima Indians (O'Dea 1991; Lillioja et al. 1993).

1.6.2 Hyperinsulinaemia and accelerated atherosclerosis

The ability of hyperinsulinaemia to compensate for insulin resistance is not without consequences. Hyperinsulinaemia stimulates the SNS, affecting the heart, vasculature and

increasing sodium retention by the kidneys, factors which increase blood pressure (Reaven *et al.* 1996). Hence, the incidence of hypertension is also high in patients with type II diabetes (40% in men and 53% in women at diagnosis (UK Prospective Diabetes Study (UKPDS) 1985)). Hyperinsulinaemia is a hallmark of hypertension, IGT and type II diabetes in the obese population, in all of whom the risk of CHD is increased (Welborn and Wearne 1979; Fuller *et al.* 1983; Pyörälä *et al.* 1985; Fontbonne *et al.* 1991). Hyperinsulinaemia may contribute to the atherogenesis, by increasing arterial wall smooth muscle cell proliferation and lipid synthesis, promoting plaque progression (Stout 1990). In addition, hyperinsulinaemia has been associated with other atherogenic factors, including elevated levels of plasminogen activator inhibitor type 1 (PAI-1), resulting in decreased fibrinolytic activity, advancing plaque progression and thrombus formation (Juhan-Vague *et al.* 1991).
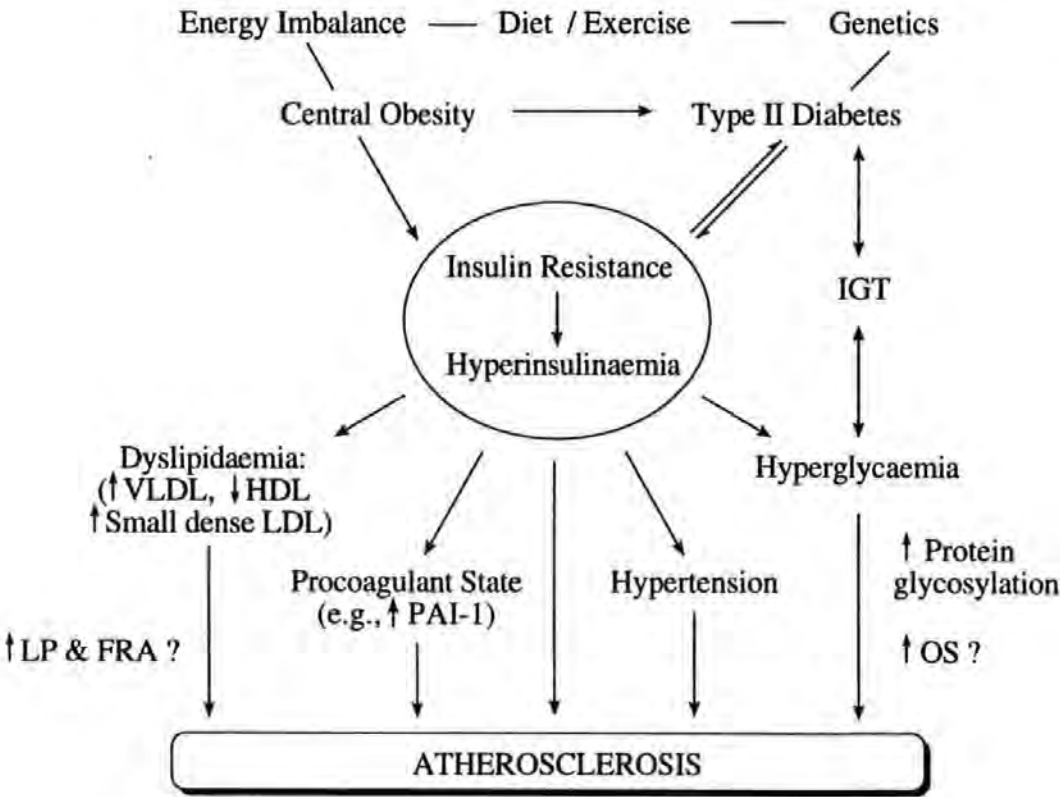
Figure 1.12. *The insulin resistance syndrome and atherogenesis. LP, lipid peroxidation, FRA, free radical activity; OS, oxidative stress; ↑, increase; ↓, decrease.*

Abdominal obesity and the insulin resistance syndrome are associated with multiple risk factors for CHD, including hypertension, raised VLDL-triglycerides, decreased HDL-cholesterol, IGT and hyperinsulinaemia (Reaven 1988). Thus, insulin resistance and hyperinsulinaemia are closely interrelated and appear to have a central role in the development of atherogenesis in type II diabetes (Figure 1.12). However, diabetes *per se* is known to confer an additional risk of developing CHD (Garcia *et al.* 1974; Stamler *et al.* 1993). The additional factors involved in atherogenesis in diabetes have been reviewed by Bierman (1992) and include, alterations in coagulation factors, forming a procoagulant state, enhanced activity of growth factors and cytokines and protein glycation.

Recently, attention has focussed on the role of free radical activity in the development of atherosclerosis. It is accepted that the oxidation of LDL is a key stage in the development of foam cells, although the exact mechanisms which initiate lipid peroxidation and modification of the LDL apoproteins are uncertain (Berliner and Heinecke 1996). In diabetes, evidence has been accumulating which suggests that circulating LDL is more sensitive to oxidation and may already be undergoing oxidative modification prior to entering the arterial wall (Babiy *et al.* 1992; Bowie *et al.* 1993; Beaudeux *et al.* 1995). Additionally, the susceptibility of LDL to *in vitro* oxidation from type I diabetic patients with poor glycaemic control was increased (Tsai *et al.* 1994), whilst LDL from patients with well controlled type I diabetes was not more susceptible to oxidation (Jenkins *et al.* 1996). Increased amounts of small dense LDL, which is also more readily oxidized have been reported in patients with type II diabetes (Peeples *et al.* 1989; Selby *et al.* 1993). These changes arise from disturbances in lipid metabolism associated with abdominal obesity, insulin resistance and glycation of the apoproteins (Bierman 1992; Reaven *et al.* 1993b).

Elevated levels of lipid peroxides have been found in patients with CHD (Ledwozyw *et al.* 1986; Stringer *et al.* 1989) and diabetes (Appendix 3). Several studies have found that lipid peroxides are also increased in persons with hyperlipidaemia, abdominal obesity and IGT (Chirico *et al.* 1993; Van Gaal *et al.* 1995; Niskanen *et al.* 1995). Thus, increased free radical activity may explain the high incidence of CHD in persons with obesity, IGT and in

particular, type II diabetes, the most common condition associated with increased oxidative stress and accelerated atherosclerosis.

1.6.3 The effect of weight loss

Obesity and, hence, diet and exercise, are important environmental determinants in the pathogenesis of type II diabetes. Weight loss has been demonstrated as the treatment of choice for obese type II diabetic patients, as it reverses the metabolic syndrome of insulin resistance by improving insulin secretion, increasing insulin sensitivity, improving glucose tolerance, lipid metabolism and reducing hepatic glucose output (Doar 1975; Hughes *et al.* 1984; Henry *et al.* 1986a). Indeed, weight loss can have a dramatic effect as reported by O'Dea (1984); Aborigines who returned to the 'hunter-gatherer' lifestyle for a short period (7 weeks), experienced weight loss, with marked improvements in glucose tolerance, normalization of plasma lipids and reversal of type II diabetes. Even caloric restriction or modest weight losses (> 6.9-10 kg) are often sufficient to improve glycaemic control with long term benefits (Henry *et al.* 1986b; Freidenberg *et al.* 1988; Wing *et al.* 1987a; Rotella *et al.* 1994). One study has found that each 1 kg of weight loss, over the first year after diagnosis, was associated with a 3-4 month increase in life expectancy in patients with type II diabetes, whilst a 10 kg weight loss could result in a 35% improvement in life expectancy (Lean *et al.* 1990).

Unfortunately, weight loss is seldom achieved and poorly sustained with conventional low calorie diets (800-1500 kcal/day) (West 1973; UKPDS 1983) and treatment of hyperglycaemia with sulphonylurea or insulin is associated with further weight gain (UKPDS 1995). Hence, obese diabetic patients have been described as "notoriously resistant to treatment" and gastric by-pass surgery has been advocated as the only effective long term therapy for subjects with a BMI > 35 (Pories *et al.* 1995).

Very low calorie diets (VLCDs) have also been used as an aggressive therapy to produce rapid weight loss in obese subjects, whilst preserving vital lean body mass (Wing 1992).

The first VLCDs were used in the 1920s, to produce larger and more rapid short-term weight loss than low calorie diets, whilst avoiding the dangers of total starvation. Today's VLCDs are specifically formulated to provide 400-800 kcal/day, with enriched amounts of high quality protein (45-100 g/day), a full complement of vitamins, trace elements and fatty acids, but not calories. Typical diets are produced in the form of powders to be mixed with water, forming soups, milk-shakes or desserts, in order to replace completely normal food intake. Such diets are normally administered for 12-16 weeks, as part of medically supervised weight loss programmes, producing on average, weight losses of 1-2 kg per week or 20 kg over 12 weeks (National Task Force on the Prevention and Treatment of Obesity (NTFPTO) 1993; Kanders and Blackburn 1994).

A number of studies using VLCDs for the treatment of obese type II diabetic subjects have been reported (reviewed by Wing 1992; NTFPTO 1993). The short term studies, duration of 6 months or less, have shown that significant weight losses were accompanied by marked improvements in glycaemic control, blood pressure and serum lipoproteins (Amatruda et al. 1988; Uusitupa et al. 1990b). Glycaemic control improved dramatically, within 7-10 days of starting these diets and required adjustment of hypoglycaemic drug therapy (Henry et al. 1985; Wing et al. 1987a; Rotella et al. 1994). The long term studies have shown that the most favourable results were obtained when VLCDs were combined with behavioural therapy (Wadden and Stunkard 1986; Wadden et al. 1989; Wing et al. 1991). Whilst VLCDs achieved large weight losses, weight regain was the main problem. After 5 years, almost all of the patients had regained all of the weight lost, regardless of which therapy was used (Wadden et al. 1989). However, improvements in glycaemic control were sustained even after most of the weight loss was regained (Wing et al. 1991).

Research is continuing into the long term effects of VLCDs and since the prevalence of obesity is increasing, the use of VLCDs for the treatment of obesity may increase in the future. As there have been no reports of the effects of such diets on oxidative stress in patients with type II diabetes, this provided the impetus for investigating these factors in this thesis.

1.7 Summary and Aims

Type I and type II diabetes are distinct conditions which share a striking propensity to both micro- and macrovascular complications. Macrovascular disease is almost certainly multifactorial in its causation. Hyperlipidaemia, hyperglycaemia, hypertension and lipid peroxidation have all been implicated in the process and insulin resistance / hyper-insulinaemia are also tightly linked to atherosclerosis and type II diabetes mellitus. Lipid peroxidation, by the action of free radicals, plays a key role in the oxidation of LDL during the early stages of atherosclerosis (Steinberg et al. 1989) and the extensive studies of Esterbauer et al. (1992) have shown that antioxidants are important in preventing this oxidative damage. Current research is focussed on the role of free radicals in the pathogenesis of atherosclerosis and on the important preventative role of dietary antioxidants.

Free radicals are formed during normal cellular metabolism, their production being counterbalanced by the action of antioxidant mechanisms. The formation and removal of free radicals is a dynamic process and under normal cellular conditions a low steady state of ROS would be reached. Hence, any condition which leads to a disturbance in the pro-oxidant / antioxidant balance in favour of pro-oxidation, results in oxidative stress with potentially damaging consequences, such as lipid peroxidation (Sies 1991).

Type II diabetes is the most common condition associated with increased oxidative stress and accelerated atherosclerosis and it has been proposed that oxidative stress contributes to the development of diabetic complications (Wolff 1987; Baynes 1991; Giugliano et al. 1996). Numerous studies have confirmed the presence of elevated markers of free radical activity in persons with type II diabetes, with and without complications, supporting the hypothesis that oxidative stress is increased in diabetes (Appendix 3). However, the role and origin of oxidative stress is less clear. The detection of markers of oxidative stress, such as elevated levels of lipid peroxides, are not sufficient within themselves to implicate oxidative stress in the pathogenesis of diabetic complications, since oxidative stress may occur secondary to the tissue damage. Thus, initially, it must be shown that oxidative stress results in tissue damage, leading to diabetic complications and secondly, that inhibition of

oxidative stress, by antioxidant therapy, slows or prevents the disease process (Baynes 1991). Oxidative stress, in diabetes, may occur as a result of a decrease in antioxidant defences due to an inadequate dietary supply or disturbances in the metabolism of vitamins, or as a result of an increase in the endogenous formation of free radicals, which overwhelm the antioxidant defences, or both.

The sources of free radicals thought to be responsible for the elevated levels of lipid peroxides found in diabetes include:

- **The respiratory burst of phagocytic cells.** Increased $O_2^{\cdot-}$ production, by leucocytes, has been reported in diabetic patients and also in subjects with hypertriglyceridaemia (Kitahara *et al.* 1980; Shah *et al.* 1983; Hiramatsu and Arimori 1988), coupled with a reduction in the activity of SOD (Nath *et al.* 1984).

- **The vascular endothelium and altered prostanoid production.** Superoxide free radicals may be generated by the endothelium during the activation of cyclo-oxygenase enzymes, stimulated by hyperglycaemia (Cohen 1993; Tesfamariam 1994), or by xanthine oxidase activity during tissue ischaemia and reperfusion (McCord 1985; Bulkley 1994). The interaction of $O_2^{\cdot-}$ with NO (EDRF) may also lead to the formation of $ONOO^-$ and other ROS.

- **The polyol pathway and pseudohypoxia.** The conversion of glucose to sorbitol by aldose reductase consumes NADPH, which is necessary for the reduction of GSSG to GSH by glutathione reductase and the recycling of antioxidants, resulting in decreased resistance to oxidative stress (Nagasaka *et al.* 1989). Imbalances in the intracellular ratio of NADH/NAD+, caused by hyperglycaemia, mimic the effects of true hypoxia (pseudo-hypoxia) and may lead to an increase in the formation of $O_2^{\cdot-}$ (Williamson *et al.* 1993).

- **Lowering of antioxidant defences.** Diabetes may cause a lowering of GSH, SOD, catalase and vitamins A and C, enabling free radical production to increase above basal levels (Costagliola 1990; Will and Byers 1996; Sundaram *et al.* 1996).

- **Glycoxidation / autoxidation reactions of glucose** (Baynes 1991; Wolff 1993).

Numerous *in vitro* studies have shown that the non-enzymatic autoxidation reactions of glucose are sources of ROS, which may potentiate protein damage by increasing crosslinking, fragmentation and the formation of AGEs (Hunt and Wolff 1991; Hunt *et al.* 1993). Glycated proteins also undergo oxidative reactions with the formation of free radical intermediates Since proteins are found in close proximity to lipids, the generation of ROS can potentially initiate the autocatalytic reactions of lipid peroxidation (Kawamura *et al.* 1994). Consequently in diabetes, hyperglycaemia may enable an increase in the endogenous formation of free radicals and constitute a starting point for oxidative stress (Hunt and Wolff 1991; Wolff *et al.* 1991; Wolff 1993). Despite confirmation from numerous *in vitro* studies that the autoxidation / glycoxidation reactions of glucose are sources of ROS, clear evidence that these reactions occur *in vivo* is lacking. A preliminary investigation of this hypothesis were, therefore, undertaken during the development of the HPLC methodology. **The effects of acute episodes of hyperglycaemia on lipid peroxidation and antioxidant vitamin status, in patients with poor glycaemic control and diabetic ketoacidosis, were investigated as part of this study.**

Obesity is a major factor in the development of disorders such as CHD and predisposes to the development of type II diabetes. Weight reduction, therefore, plays an important part in the treatment of obese diabetic patients. Very low calorie diets have been used for the treatment of obese subjects and are being increasingly used for the treatment of obese type II diabetic patients. The reduction in weight produces improvements in glycaemic control, serum cholesterol, triglycerides and other cardiovascular risk factors (Wing 1992). Low calorie / low fat diets may affect vitamin A and E intakes with the potential of compromising antioxidant protection. The possibility exists that diabetic patients may be under increased oxidative stress whilst on a VLCD, as there have been no reports of vitamin status and free radical activity in diabetic patients on such diets. **This study, therefore, aimed to compare the safety and efficacy of a VLCD with a conventional, but intensive, weight loss programme. A clinic was set up in order to monitor closely diabetic patients in the study. The effects of a VLCD on cardiovascular risk factors and indices of oxidative stress were measured.**

The aims of this study were:

1. To develop the HPLC methodology to measure:

    i) lipid peroxidation in plasma, using the TBA test and the conjugated-diene isomer of linoleic acid, as indirect indicators of free radical activity;

    ii) the plasma concentrations of vitamins A, C and E, as indicators of antioxidant status.

2. Then using these methods to:

    i) investigate the effect of very poor glycaemic control, resulting in ketoacidosis, on lipid peroxidation and antioxidant vitamin status;

    ii) study the effects of a VLCD and an intensive weight loss programme, on cardiovascular risk factors and indices of oxidative stress in obese subjects and assess the effects of improved glycaemic control on free radical activity.

This thesis is divided into two main sections: section 1, consisting of chapters 2-5 details the methods that were developed for the measurement of plasma vitamins A, C, and E and lipid peroxidation in plasma (MDA and conjugated dienes); section 2 consisting of chapters 6-7 details the studies that were carried out on diabetic patients.

Chapters 2-5

Measurement of Lipid Peroxidation and Antioxidant Vitamins in Plasma

2. Measurement of the Malondialdehyde-Thiobarbituric Acid Adduct in Plasma by HPLC

2.1 Introduction

In this study, lipid hydroperoxides and MDA in plasma samples were measured by the HPLC method of Wong *et al.* (1987) with fluorimetric detection (Young and Trimble 1991). The lipid hydroperoxides were hydrolysed in acidic conditions to yield MDA, heated with TBA reagent, the proteins were removed by precipitation and centrifugation and the MDA-TBA adduct of the resulting extract chromatographed.

2.2 Equipment

The HPLC equipment consisted of a series 2 pump and an R-100 chart recorder (Perkin Elmer Ltd., Buckinghamshire, UK), a Shimadzu RF-535 fluorescence monitor (Dyson Instruments Ltd., Tyne and Wear, UK). A Model 7125 manual injector (Rheodyne, Macclesfield, Cheshire, UK), equipped with a 20 µl loop was used for sample injection. A Spherisorb 5 µm ODS-2 column, dimensions 25 cm x 0.46 cm, was used as the main analytical column, this was preceded by a 5 cm guard column containing 10 µm ODS (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). A BT3 heating block (Grant Instruments Ltd., Cambridge, UK) was used to heat the samples.

2.3 Chemicals and Reagents

Orthophosphoric acid (specific gravity 1.7, 85%), sodium hydroxide, disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4.2H_2O$), sodium dihydrogen orthophosphate dihydrate ($NaH_2PO_4.2H_2O$), all A.R. grade were obtained from BDH., Merck Ltd., Poole, Dorset, UK. Ethanol (99.7%) was obtained from Hayman Ltd., Witham, Essex, UK. Methanol (HPLC grade) was obtained from Rathburn Chemicals Ltd., Walkerburn, Scotland. Thiobarbituric acid (TBA) reagent (4,6-dihydroxypyrimidine-2-thiol) and 1,1,3,3-tetra-ethoxypropane (TEP), purity 97%, were obtained from Sigma Chemical Co., Dorset, UK. Distilled water was obtained from the Pharmacy Manufacturing Unit at Torbay Hospital for the preparation of all solutions.

TEP standard solutions

Stock solutions of TEP (8.1 mmol/l) were prepared by diluting 0.5 ml of the TEP reagent to 250 ml with ethanol in water (40% v/v). An intermediate standard solution (40.5 μmol/l), was prepared by diluting 0.5 ml of the TEP stock solution to 100 ml with water. Working standard solutions with concentrations of 0.61, 1.22, 2.43 and 4.86 μmol/l were prepared by diluting 3 ml of the intermediate standard solution to 200, 100, 50 and 25 ml, with water, respectively.

A 0.6% (w/v) solution of TBA, in water, was prepared for each analytical run. Phosphoric acid (1.22 mol/l) was prepared by diluting 14 g of the concentrated acid to 100 ml with water.

Mobile phase

A phosphate buffer solution (25 mmol/l), pH 6.5, was prepared by dissolving 1.42 g $Na_2HPO_4.2H_2O$ and 2.66 g $NaH_2PO_4.2H_2O$ in 1l of water. The mobile phase was prepared by mixing methanol and buffer in a 1:1 ratio by volume. The mobile phase was degassed with helium for 5 minutes before use and was delivered isocratically with a flow rate of 1 ml/minute.

Cleaning solution

A solution of TBA in acid was used to clean the reaction vessels after each analysis. This solution was prepared by mixing 300 ml of dilute phosphoric acid (10 ml of the concentrated acid diluted to 1l) with 300 ml of a 0.2% (w/v) solution of TBA.

2.4   Sample Preparation

Venous blood samples for the analysis of TBARS, the conjugated diene isomer of linoleic acid and plasma vitamins A, C and E, were collected from healthy, nonfasting volunteers, into 10 ml Vacutainer tubes containing ethylenediaminetetraacetic acid ((EDTA) Becton Dickinson Ltd., Oxford, UK). After centrifugation at room temperature (1000 x g, for 10 minutes), the supernatant plasma was removed carefully to avoid contamination with platelets and leucocytes and either analysed immediately or aliquoted and frozen at -70°C.

The TBA reaction was carried out in glass stoppered tubes by mixing 450 μl of water, 250 μl of phosphoric acid (1.22 mol/l) and 250 μl of the TBA reagent with 50 μl of either the standard, plasma sample or water as a blank. The reaction mixture was heated at 100°C for exactly 1 hour in a heating block and then kept in ice until the analysis could be performed. Immediately before injection on to the HPLC column, 200 μl of the reaction mixture were added to 40 μl of sodium hydroxide (1 mol/l) in a clean glass centrifuge tube, 360 μl of methanol were added, the sample was vortex mixed and centrifuged at 2500 x g for 2 minutes to precipitate the proteins. A 20 μl volume of the clear supernatant was then injected on to the column for analysis. The samples were analysed in duplicate, two blank samples and a series of working standards were included in each analytical run.

A cleaning procedure was adopted at the end of each analytical run. The columns were flushed with methanol / water in a 6:4 ratio by volume for 20 minutes, followed by pure methanol for a further 15 minutes. The glass tubes used for the reaction were washed and rinsed with distilled water, filled with the TBA cleaning solution, stoppered and heated for 1 hour at 100°C. The tubes were then rinsed with water, refilled with water and heated again at 100°C for 1 hour. After a final rinse, the tubes were drained and dried. These precautions were necessary in order to minimize the risk of contamination in subsequent analyses.

2.5 Method Validation

Detector response

Fluorimetric detection was carried out at excitation and emission wavelengths of 532 nm and 553 nm respectively. The linearity of the detector response was investigated by preparing and analysing a series of TEP standards with concentrations ranging from 0.5 to 10 μmol/l. By diluting the standard with the lowest concentration the minimum detectable amount was obtained (Lindsay 1992).

Precision

The within batch variation of the method was determined by repeating the analysis on a fresh plasma sample, on the same day, under identical conditions. The remainder of the plasma was stored in 150 μl aliquots at -70°C. Each analytical run contained one sample enabling the between batch variation to determined.

Accuracy

The analytical recovery was performed by adding TEP standards with concentrations of 1, 2.5 and 50 μmol/l to quadruple sets of plasma. Plasma was also analysed without the addition of standards enabling the percentage of each standard recovered to be calculated.

Stability

Fresh plasma samples were obtained and analysed on the day of collection, the remainder were stored in 150 μl aliquots at -70°C. Samples were analysed on a monthly basis to give an indication of the long term stability.

2.6   Results

Detector response

A series of standard solutions of TEP were included with every sample batch. Typical chromatograms are shown in Figures 2.1 and 2.2. The detector response was linear up to a MDA concentration of at least 10 μmol/l as indicated in Figure 2.3. The assay was sensitive to 0.2 μmol/l, although no plasma samples with such low concentrations were observed.

Precision

The within batch variation, expressed as the coefficient of variation, was 6.3% ($n = 10$) and the between batch variation was 9.9% ($n = 10$).

Accuracy

The addition of 1, 2.5 and 5 μmol/l of MDA, in the form of TEP, to plasma samples resulted in mean recoveries of 109%, 102% and 104% ($n = 4$) respectively.

Stability

The MDA concentration in two sets of plasma samples stored at -70°C is shown in Figure 2.4. The levels of MDA gradually increased in one set of plasma (plasma 2) after 5 months of storage and by 9 months the levels of MDA had increased by 28%. In a second set of plasma (plasma 1), containing a higher initial concentration of MDA, an increase of 8% was observed after 9 months of storage, which was still within the precision of the method.
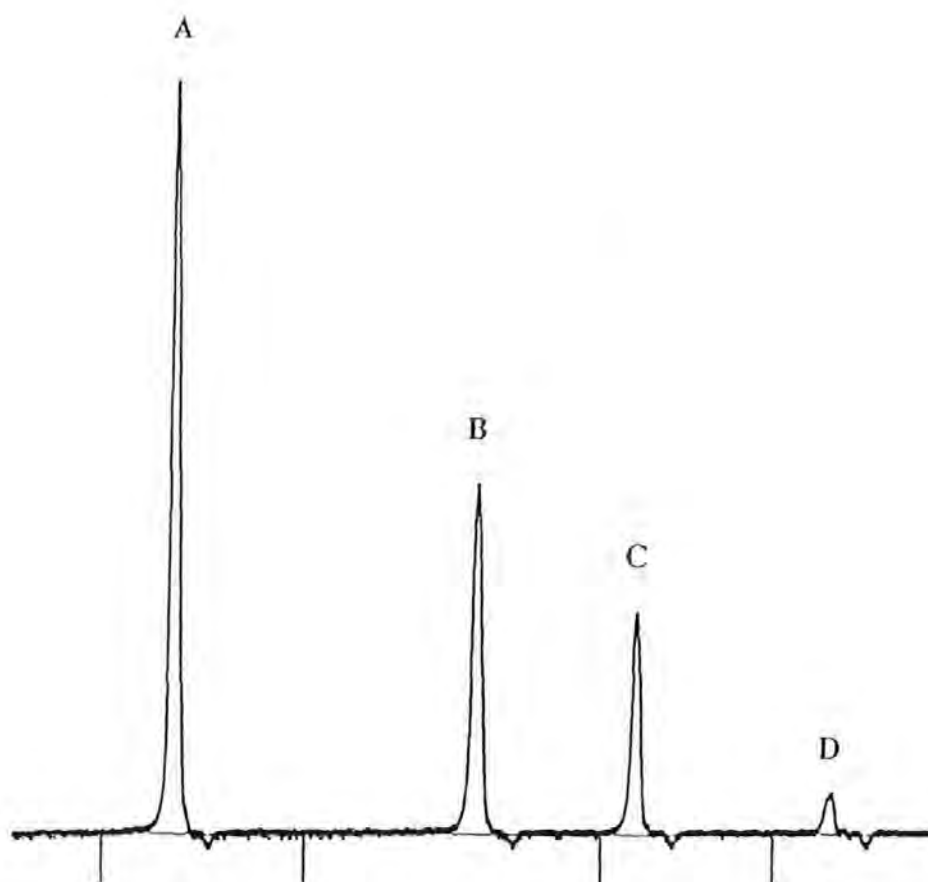
Figure 2.1 *A typical chromatogram of standard TEP solutions, A, B and C corresponding to 2.43, 1.22 and 0.61 μmol/l MDA respectively and a blank sample (D).*

3.6
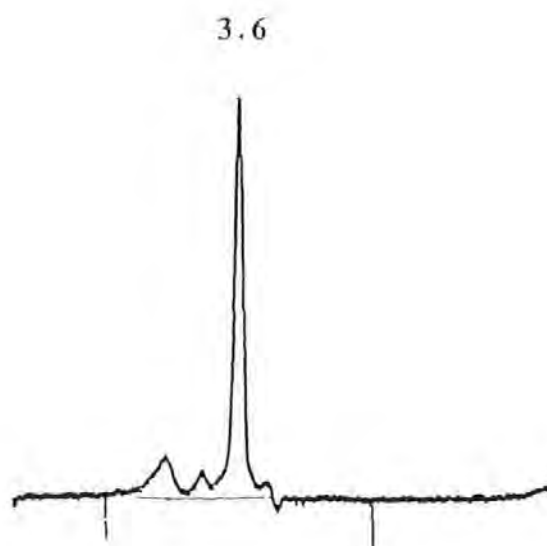
Figure 2.2 *A typical chromatogram of a plasma sample showing the peak corresponding to the MDA-TBA adduct (retention time 3.6 min).*
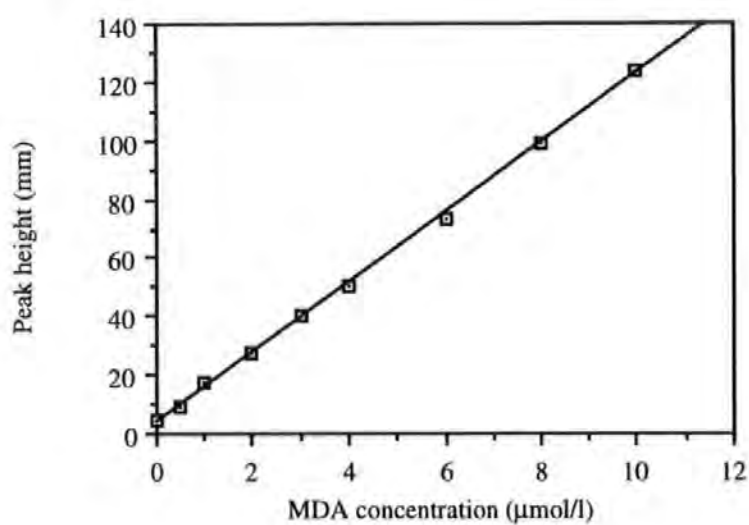
Figure 2.3 *Standard curve for MDA.*
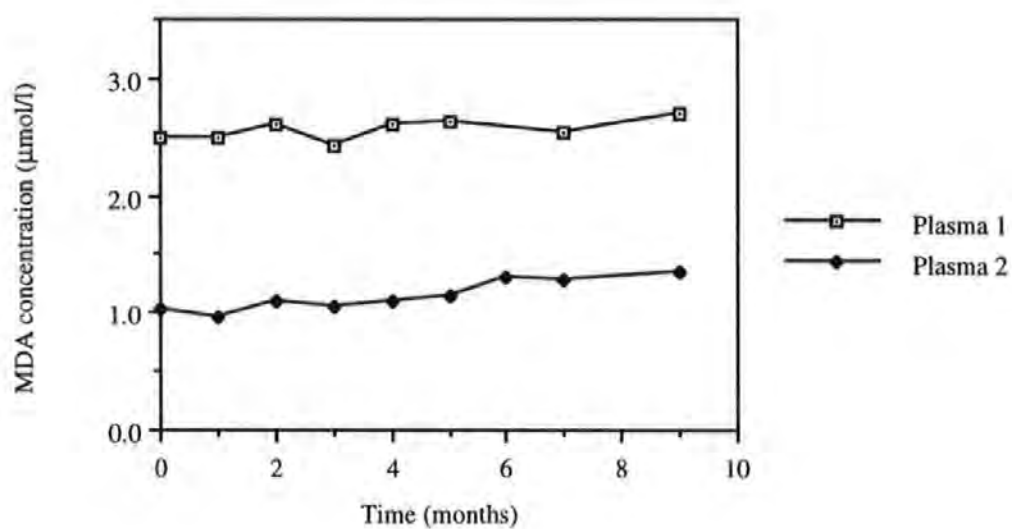
Figure 2.4 *Plasma MDA concentrations (µmol/l) in two sets of samples stored at -70˚C.*

2.7 Discussion

The determination of MDA by the reaction with TBA remains the most widely used marker of lipid peroxidation in biological samples. Several compounds including carbohydrates and amino acids have also been found to react with TBA causing interference (Esterbauer *et al.* 1982b; Knight *et al.* 1988). The use of HPLC has enabled the separation of these compounds from the true MDA-TBA adduct, thereby increasing the specificity of the assay.

The detector response was found to be linear up to at least 10 μmol/l and encompassed the concentration range expected in clinical samples. Young and Trimble (1991) reported the assay to be linear up to at least 48 μmol/l.

During the initial attempts at obtaining values for the precision of the method, contamination was found to be a frequent problem. This was reflected by a very high between batch variation of 25%. Also, the occasional inexplicable high value in plasma samples was observed, a finding confirmed by Dr Young (personal communication).

Several precautions were taken in order to minimize interferences with this assay. These included collecting blood samples in tubes containing EDTA and rejecting any samples showing signs of haemolysis. The rigorous cleaning procedure of the glassware described by Wong *et al.* (1987) was adopted to minimize the risk of contamination and to maintain the level of background interference as low as possible. Since platelets are sources of lipid peroxides, care was taken to avoid the 'buffy coat' layer whilst separating the plasma from the red cells and aliquoting the samples for analysis. Finally, the purest source of distilled water was sought and used for the preparation of all samples and solutions. With these precautions, a within batch variation of 6.3% and a between batch variation of 9.9% were obtained. These results compared favourably to those of Young and Trimble (1991), who reported a within batch variation of 6.2% and a between batch variation of 9%. Acceptable values were obtained for the accuracy of method, although poor recovery has been reported by some authors (Hackett *et al.* 1988).

The plasma range of MDA in healthy subjects varies markedly according to the method used. Simple spectrophotometric or fluorimetric methods have reported generally higher values than HPLC based methods (Table 1.5, page 37). Using this method, the plasma MDA concentration in 17 healthy subjects, aged between 26 and 67 years, was found to be 1.0 ± 0.2 μmol/l (mean $\pm$ SD). This result was higher than that reported by Wong *et al.* (1987) and Young and Trimble (1991) who reported values of 0.6 ± 0.1 μmol/l (mean $\pm$ SD).

Plasma samples collected in EDTA appeared to be stable for 5 months at -70°C. Young and Trimble (1991) reported EDTA plasma samples to be stable for 10 days at 4°C, 3 weeks at -20°C and at least 4 months at -70°C.

In summary, it was not the purpose of this work to propose critical modifications to the preparative procedure of this method, owing to the time limit of this study, but to reproduce the conditions described by Wong *et al.* (1987) and Young and Trimble (1991) and this was achieved. The assay, although simple to perform, was found to be subject to contamination from a wide variety of sources and required carefully controlled conditions to give reproducible results.

3. Measurement of the Conjugated Diene Derivative of Linoleic Acid in Plasma by HPLC

3.1 Introduction

In this study, the conjugated diene isomer of linoleic acid and its molar ratio to linoleic acid, in total plasma lipids, were measured by the HPLC method of Iversen *et al.* (1985), with the modifications developed at Southmead Hospital, Bristol (Dr. Bolton, personal communication). The concentrations of arachidonic acid, linolenic acid, palmitic acid, palmitoleic acid, oleic acid and stearic acid were also determined in the samples.

3.2 Equipment

The HPLC instrumentation consisted of a series 410 LC pump, an ISS-100 autosampler, LC-90 and LC-75 UV spectrophotometric detectors linked in series, an R-100 recorder and an LCI-100 laboratory computing integrator, all obtained from Perkin Elmer Ltd., Buckinghamshire, UK. Chromatographic separation was performed on a Hypersil 3 μm MOS, 15 cm x 0.46 cm, analytical column with a 5 cm guard column containing 5 μm ODS, at 30°C using a column heater (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). The handling of samples and solutions was carried out with the use of glass, positive-displacement micro-pipettors, Alpha Laboratories, Eastleigh, Hampshire, UK.

3.3 Chemicals and Reagents

Arachidonic acid (5,8,11,14-eicosatetraenoic acid), linoleic acid (9,12-octadecadienoic acid), linolenic acid (9,12,15-octadecatrienoic acid), palmitic acid (hexadecanoic acid), palmitoleic acid (9-hexadecenoic acid), oleic acid (9-octadecenoic acid), stearic acid (octadecanoic acid), internal standard (*cis* 11,14-eicosadienoic acid) and butylated hydroxytoluene (BHT) were obtained from Sigma Chemical Co., Poole, Dorset, UK. Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland. Chloroform, concentrated hydrochloric acid (specific gravity 1.18), glacial acetic acid, hexane, sodium hydroxide, anhydrous sodium sulphate, all A.R. grade, were obtained from BDH, Merck Ltd., Poole, Dorset, UK.

Ethanol (99.7%) was obtained from Hayman Ltd., Witham, Essex, UK. The biological isomer (9-*cis*, 11-*trans*-octadecadienoic acid) was not commercially available so the stereoisomer (9-*trans*, 11-*trans*-octadecadienoic acid) was used as the conjugated-diene standard. This was kindly provided by Dr.Wickens, Whittington Hospital, London, UK.

Standard solutions

All glassware was cleaned with concentrated hydrochloric acid (50% (v/v)) prior to use. The following stock solutions were prepared by weighing each fatty acid into a volumetric flask and diluting to the appropriate volume with ethanol containing BHT (100 mg/l) as an antioxidant. Arachidonic acid (10 mg to 10 ml), linoleic acid (0.5 g to 50 ml), linolenic acid (0.015 g to 10 ml), palmitic acid (0.25 g to 50 ml), palmitoleic acid (0.5 g to 25 ml), oleic acid (0.5 g to 50 ml), stearic acid (0.15 g to 50 ml), internal standard (0.05 g to 10ml) and the conjugated diene standard (0.7 mg to 50 ml). A working standard solution containing all the fatty acids was prepared at the time of each analytical run by combining the volumes of the stock solutions shown in Table 3.1. The final concentration of each fatty acid in the working standard solution is also shown.

Table 3.1 *Concentrations of the fatty acids in the stock and working standard solutions.*

Fatty acid stock solution	Volume used for working standard	Final fatty acid concentration in working standard solution
Arachidonic acid (3280 μmol/l)	100 μl	328 μmol/l
Linoleic acid (35650 μmol/l)	50 μl	1783 μmol/l
Linolenic acid (5390 μmol/l)	100 μl	539 μmol/l
Palmitic acid (19500 μmol/l)	250 μl	4875 μmo/l
Palmitoleic acid (78620 μmol/l)	100 μl	7862 μmol/l
Oleic acid (35400 μmol/l)	50 μl	1770 μmol/l
Stearic acid (10545 μmol/l)	300 μl	3163 μmol/l
Internal standard (16200 μmol/l)	50 μl	810 μmol/l
Total volume in working standard	1 ml	

A 20 μl portion of this working standard solution was directly chromatographed. The stock internal standard solution (16200 μmol/l) was diluted with ethanol in a 1:1 ratio before addition to plasma, during the sample preparation. The conjugated diene of linoleic acid was not commercially available, therefore, the stereoisomer was used as a standard. It was also used as an internal standard to account for losses during the sample preparation and was added directly to plasma where it acted as an internal / external standard.

Mobile phase

The mobile phase consisted of acetonitrile / water (containing 1.5 ml/l glacial acetic acid as an ion suppressant) in a 72:28 ratio by volume (v/v). The mobile phase was delivered isocratically with a flow rate of 1.5 ml/minute and degassed continuously with helium, in order to prevent baseline drift which was found to occur during the detection of the fatty acids at 210 nm.

3.4 Sample Preparation

To 250 μl of plasma in a glass stoppered tube, 250 μl of the conjugated diene standard (50 μmol/l), 25 μl of eicosadienoic acid internal standard (8100 μmol/l) and 50 μl BHT (100 mg/l in ethanol) were added. The lipids were extracted from the plasma by the addition of 5 ml of a mixture of chloroform and methanol (2:1 v/v), the tubes were flushed with nitrogen, stoppered and mixed for 10 minutes. The mixture was then filtered and the resulting solution evaporated to dryness under a constant stream of nitrogen. In order to saponify the lipids, 500 μl of methanolic sodium hydroxide (5 g / 250 ml methanol) were added, the tubes were flushed with nitrogen and the samples heated at 70°C for 35 minutes. Three drops of concentrated hydrochloric acid were added to acidify the samples and approximately 5 mg of anhydrous sodium sulphate to remove all traces of water, 5 ml of hexane were then added and the tubes were flushed with nitrogen, stoppered and mixed for 2 minutes in order to extract the fatty acids. The samples were then centrifuged at 150 x *g* for 2 minutes, the hexane layer was removed, evaporated to dryness and the residue reconstituted in 250 μl of mobile phase. A 20 μl sample was then chromatographed.

3.5 Method Validation

Detector response

The conjugated dienes and fatty acids were monitored simultaneously using two UV detectors linked in series and set at wavelengths of 234 nm and 210 nm respectively. The linearity of the detector response was evaluated by preparing and analysing a series of standards of each fatty acid and of the conjugated diene.

Extraction and saponification

The extraction of lipids from plasma using the chloroform / methanol mixture and saponification at 70°C were initially investigated in order to optimize the conditions of the sample preparation. The extraction of the lipids was assessed on a fresh plasma sample. Internal standards were added to 250 μl aliquots of plasma in glass stoppered tubes, 5 ml of chloroform / methanol (2:1 v/v) were then added and the tubes flushed with nitrogen. The lipids were then extracted from the plasma by mixing for either 1, 3, 5, 10 or 20 minutes; samples were prepared in triplicate for each extraction time. After filtering and drying the extracts under nitrogen, 5 ml of methanolic sodium hydroxide were added and the samples heated at 70°C for 30 minutes. The remaining stages were then carried out as described previously in the sample preparation.

The optimum time for saponification and release of free fatty acids at 70°C was also investigated. Internal standards were added to 250 μl aliquots of plasma and the lipids extracted with chloroform / methanol for 10 minutes under nitrogen. After filtering and drying the extracts, 5 ml of methanolic sodium hydroxide were added and the extracts heated at 70°C for either 20, 25, 30, 35, 40 or 45 minutes. The remaining stages of the sample preparation were then carried out and samples were prepared in duplicate for each time.

Precision and stability

The within batch variation of the method was determined after the extraction and saponification times had been decided. A fresh plasma sample was obtained and the

analysis repeated on the same day under identical conditions. The remainder of the plasma was frozen in 300 µl aliquots at -70°C. Each analytical run included one aliquot which acted as a quality control sample and enabled the between batch variation to be calculated. The remainder of the plasma was also analysed over a period of 12 months and the results were used to provide an indication of the stability of the fatty acids stored at -70°C.

Accuracy

The fatty acid concentration of plasma was determined with and without the addition of standard solutions of linoleic, palmitoleic and arachidonic acid with concentrations of 220, 950 and 66 µmol/l respectively. This was repeated four times on the same plasma and the percentage of each fatty acid recovered was calculated.

3.6 Results

A typical chromatogram of a working standard mixture of fatty acids is shown in Figure 3.1. The order of elution was established by running each pure standard solution singly and noting the retention time. A chromatogram of a plasma sample is shown in Figure 3.2. The components in the plasma sample were identified by comparing their retention times with those in the standard mixture and by calculating and comparing the capacity factors of the components in the samples with those in the standard. The capacity factor (k') given by, $k' = (t-t_0 / t_0)$, where t = the retention time of the component and t_0 = the retention time of an unretained peak and the ratio of capacity factors k_2' / k_1', were also used for peak identification. Where several fatty acids were chromatographed closely together, further confirmation of peak identity was achieved by the addition of small amounts of pure standard solutions to the samples. A typical chromatogram of a plasma sample showing the main conjugated diene of linoleic acid and the added internal /external conjugated diene standard is shown in Figure 3.4. The order of elution was established by preparing plasma with and without the addition of the conjugated diene standard and also by running the pure standard separately. The conjugated diene standard (9-*trans*, 11-*trans*-octadecadienoic acid) was found to elute after the plasma conjugated diene (9-*cis*, 11-*trans*-octadeca dienoic acid).
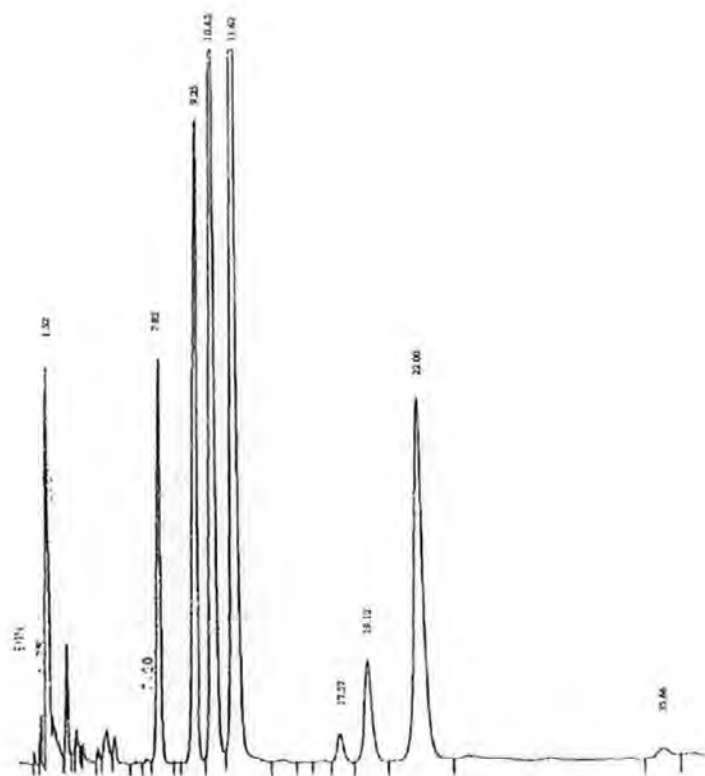
Figure 3.1 *A typical chromatogram of a standard mixture of linolenic acid (retention time 7.85 min), palmitoleic acid (9.25 min), arachidonic acid (10.45 min), linoleic acid (11.62 min), palmitic acid (17.57 min), oleic acid (19.12 min), internal standard (22.0 min) and stearic acid (35.66 min).*
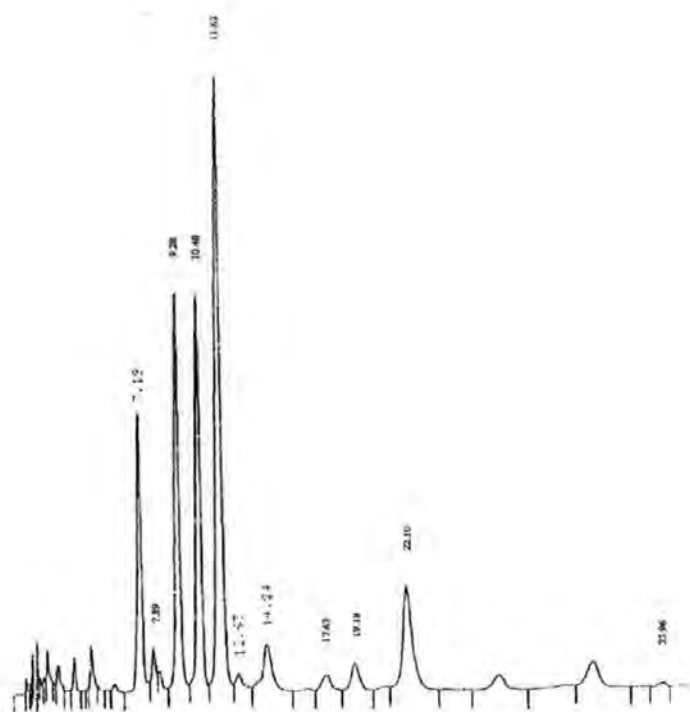
Figure 3.2 *A typical chromatogram of a plasma sample showing linolenic acid (retention time 7.89 min), palmitoleic acid (9.28 min), arachidonic acid (10.48 min), linoleic acid (11.62 min), palmitic acid (17.63 min), oleic acid (19.19 min), internal standard (22.1 min) and stearic acid (35.96 min). The detection wavelength was 210 nm at 0.01 AUFS.*
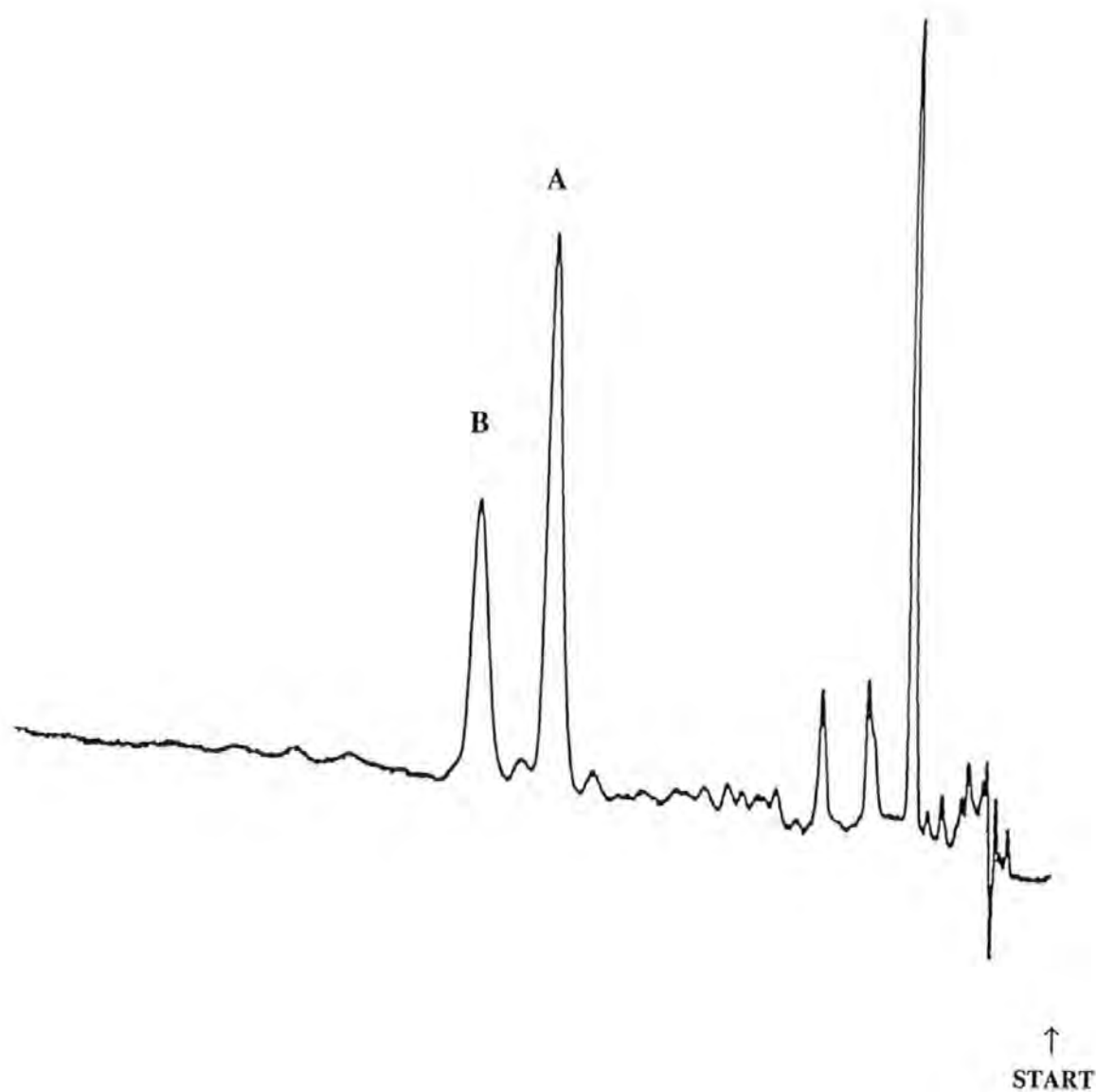
Figure 3.3 *A typical chromatogram of a plasma sample showing the principal conjugated diene, 9-cis, 11-trans-octadecadienoic acid (A) and the internal / external conjugated diene standard, 9-trans, 11-trans-octadecadienoic acid (B). The detection wavelength was 234 nm at 0.05 AUFS.*
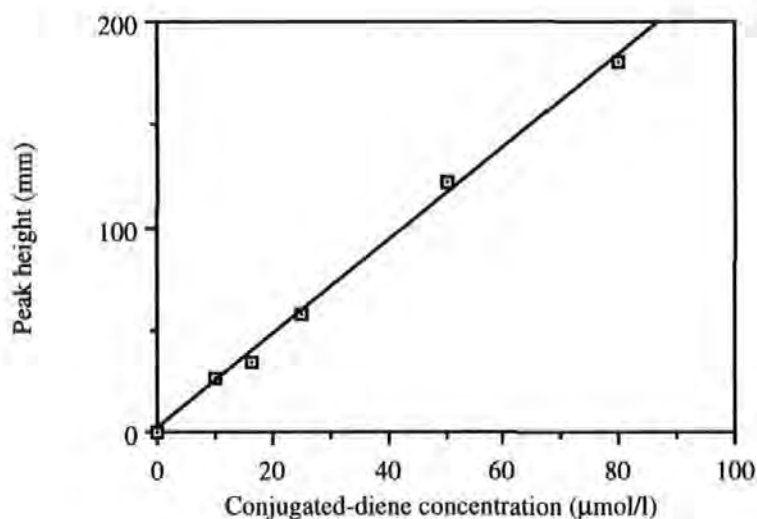
Detector response

Figure 3.4 *Standard curve for the conjugated diene of linoleic acid.*

The detector response was found to be linear to a concentration of at least 80 μmol/l for the conjugated diene standard as shown in Figure 3.4. The minimum detectable amount was the amount of analyte present in a 20 μl injection volume giving a peak whose height was twice that of the baseline noise. This was found to be 1.85 μmol/l for the conjugated diene. For the fatty acids, the detector response was linear up to at least 1600 μmol/l for arachidonic acid, 3000 μmol/l for linoleic acid, 3000 μmol/l for linolenic acid, 6300 μmol/l for oleic acid, 4500 μmol/l for palmitic acid, 8000 μmol/l for palmitoleic acid and 2500 μmol/l for stearic acid.

Extraction and saponification

Figures 3.5 to 3.8 illustrate the effect of different extraction times using the chloroform and methanol mixture (2:1 v/v) on the recovery of fatty acids from plasma.
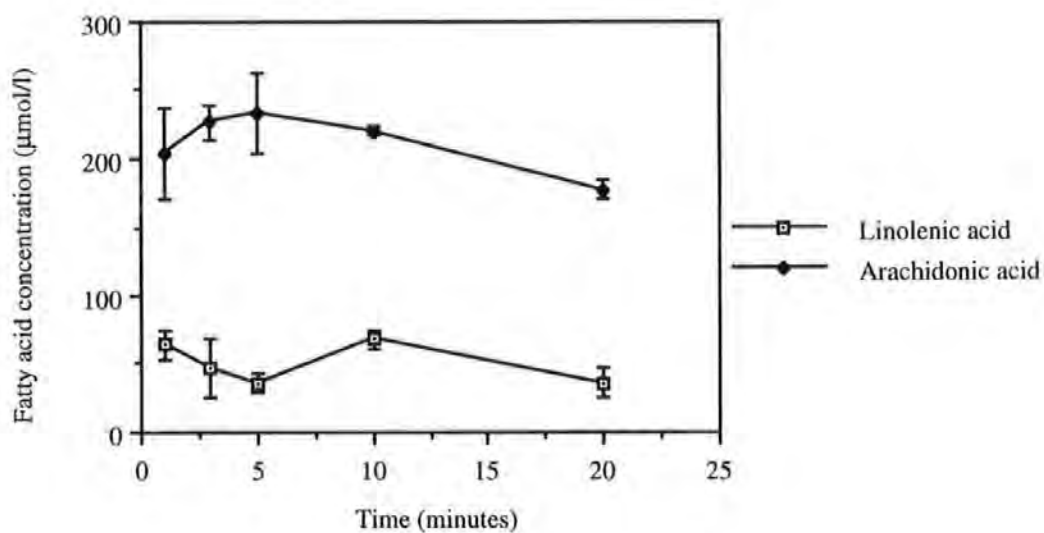
Figure 3.5 *Effect of extraction time on the concentration of linolenic and arachidonic acid (mean ± SD).*
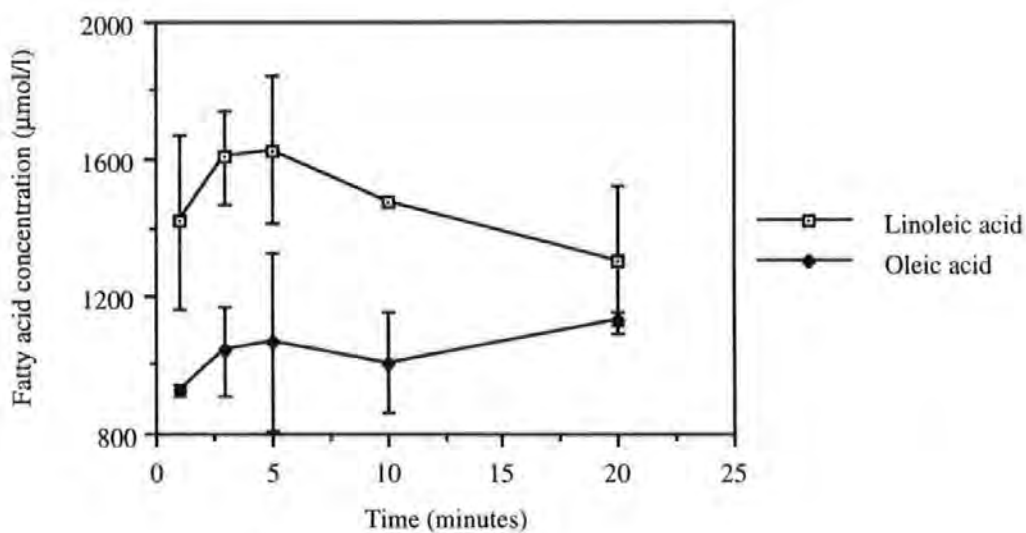
Figure 3.6 *Effect of extraction time on the concentration of linoleic and oleic acid (mean ± SD).*
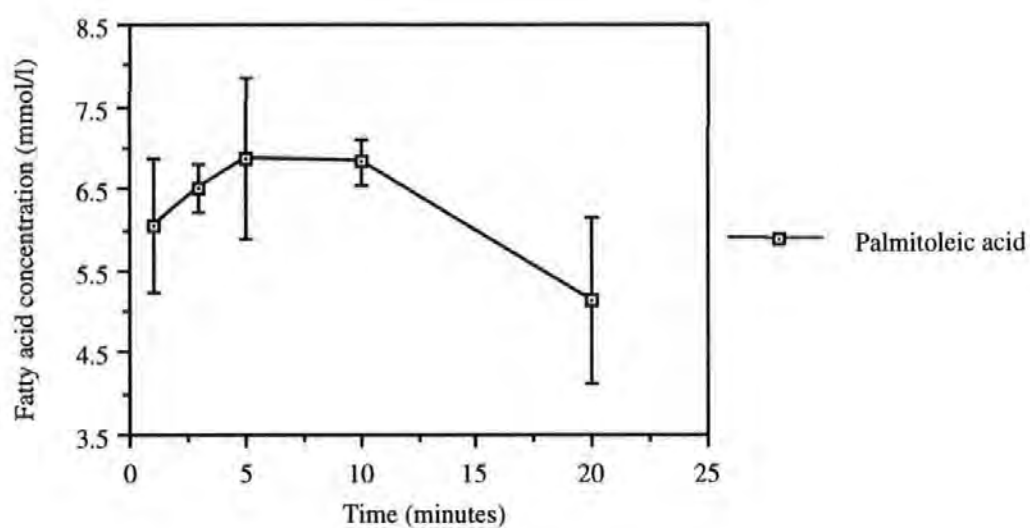
Figure 3.7 *Effect of extraction time on the concentration of palmitoleic acid (mean ± SD).*
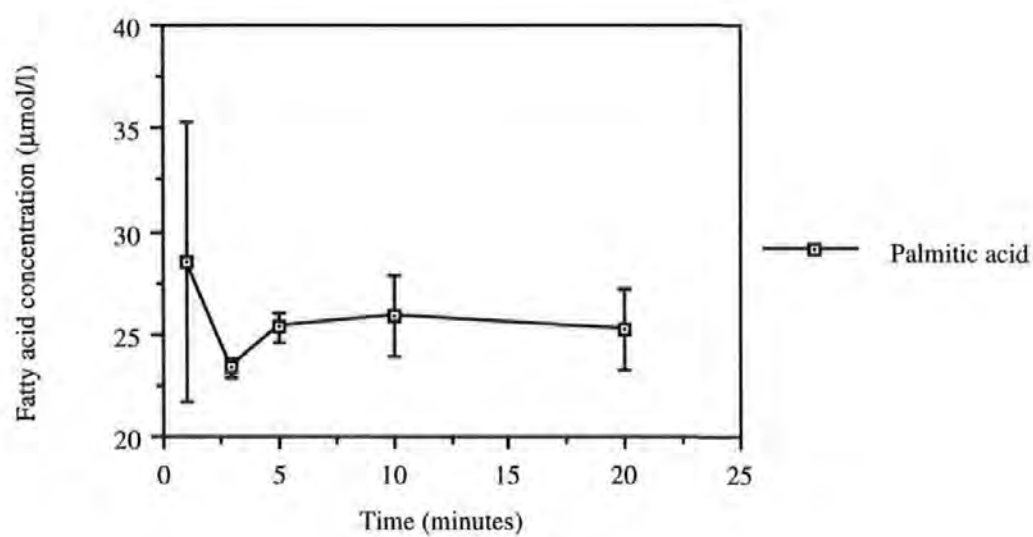
Figure 3.8 *Effect of extraction time on the concentration of palmitic acid (mean ± SD).*

95

A large variation in the results was found when extraction times of less than 10 minutes were used. The recoveries of palmitoleic acid, palmitic acid and arachidonic acid were maximum at 10 minutes, whereas the recoveries of linolenic acid, linoleic acid and oleic acid were beginning to decrease at this time. However, minimum variation in the results of four fatty acids were observed after 10 minutes and this extraction time was chosen for further work.

After the extraction time had been decided, the optimum time for saponification of the fatty acids at 70°C was investigated. The mean results for each time are presented in Figures 3.9 to 3.12. A gradual increase in the concentrations of palmitoleic, linolenic, linoleic and oleic acids were observed with increasing incubation time at 70°C. Maximum concentrations were obtained between 30 and 35 minutes, after this time the concentrations gradually declined. An unusual result was obtained for palmitic acid with an apparent decrease in concentrations from 25 to 35 minutes which was followed by an increase to a maximum value at 45 minutes. The heating time of 35 minutes was chosen for all further work as this appeared to be the optimum time for linoleic acid, the main fatty acid of interest.
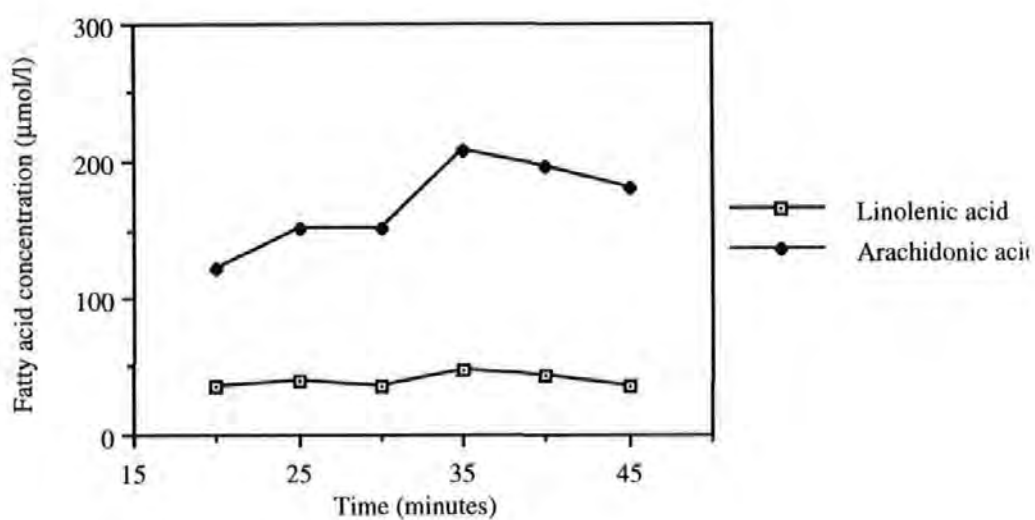
Figure 3.9 *Effect of saponification time at 70°C on the concentrations of linolenic and arachidonic acid.*
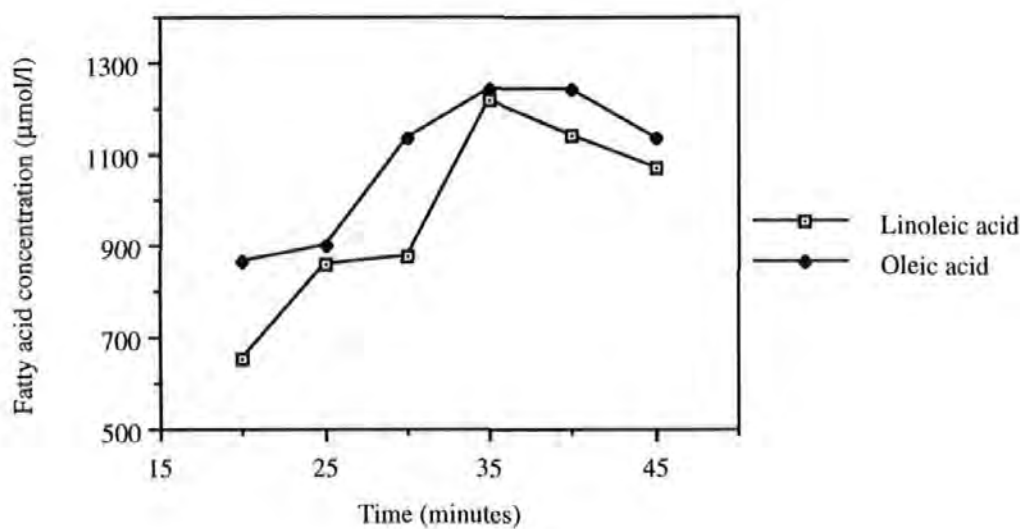
Figure 3.10 *Effect of saponification time at 70°C on the concentrations of linoleic and oleic acid.*
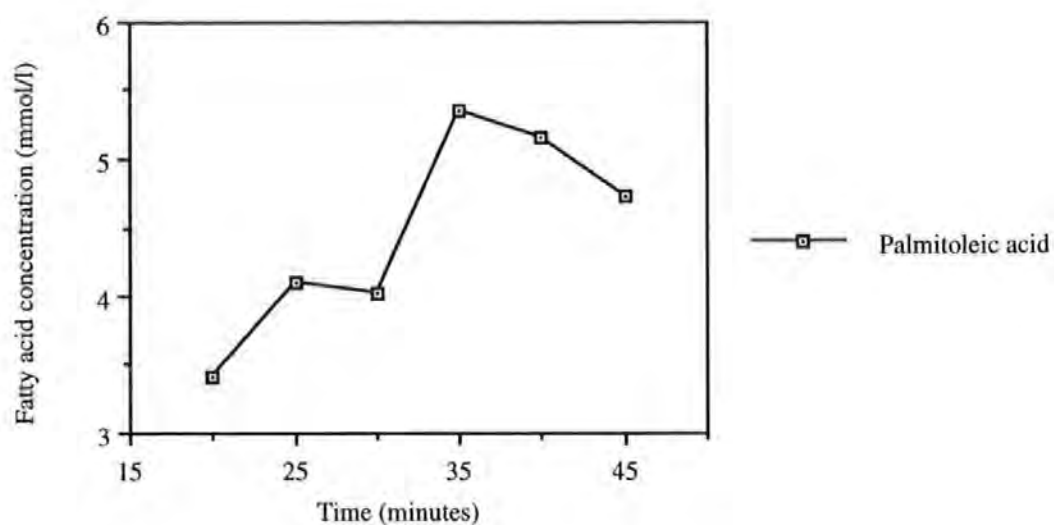
Figure 3.11 *Effect of saponification time at 70°C on the concentration of palmitoleic acid.*
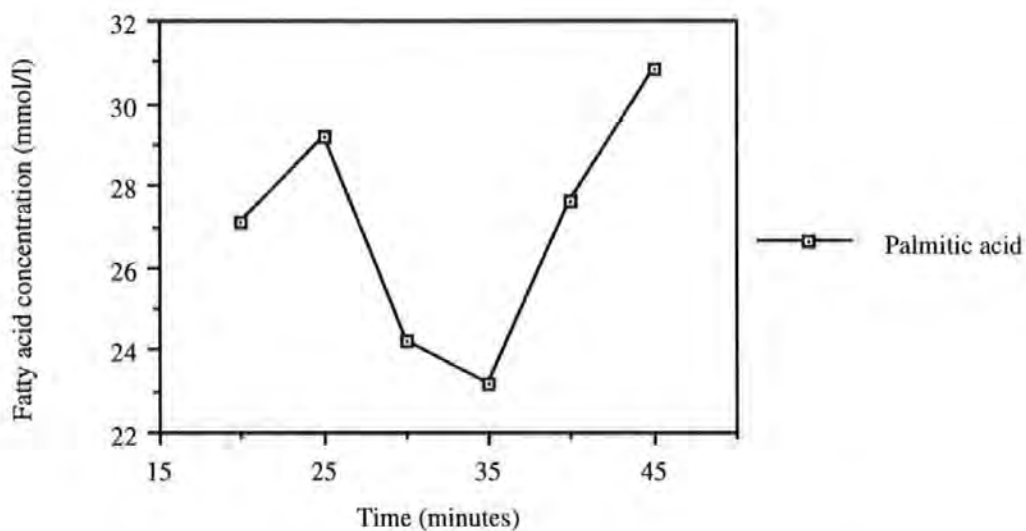
Figure 3.12 *Effect of saponification time at 70°C on the concentration of palmitic acid.*

Precision

The coefficient of variation for the within batch variation was found to be 4.3% ($n = 5$) for the conjugated diene of linoleic acid, 6% ($n = 5$) for linoleic acid, 6% ($n = 5$) for linolenic acid, 6% ($n = 5$) for palmitoleic acid, 6% ($n = 5$) for arachidonic acid, 15% ($n = 5$) for palmitic acid, 15% ($n = 5$) for oleic acid and 20% ($n = 5$) for stearic acid. The between batch coefficient of variation was 5% ($n = 7$) for the conjugated diene of linoleic acid, 9% ($n = 7$) for linoleic acid, 12% ($n = 7$) for linolenic acid, 8% ($n = 7$) for palmitoleic acid, 7% ($n = 7$) for arachidonic acid, 15% ($n = 7$) for palmitic acid, 20% ($n = 7$) for oleic acid, 25% ($n = 7$) for stearic acid and 10% ($n = 7$) for the ratio of the conjugated diene to linoleic acid.

Accuracy

The addition of 220 µmol/l of linoleic acid, 950 µmol/l of palmitoleic and 66 µmol/l of arachidonic acid to plasma resulted in recoveries of 92%, 92% and 93% ($n = 4$) respectively.

Stability

After 12 months of storage at -70°C an increase of 27% was observed in the concentration of the conjugated-diene which corresponded to an increase of 25% in the conjugated-diene / linoleic acid ratio. After 18 months of storage the concentration of the conjugated-diene had increased by 89% and the ratio had increased by 108%.

3.7 Discussion

Some initial difficulties were encountered with this method. These included large variations in the retention times between sample injections, making it very difficult to identify the components of the chromatograms. The problem was resolved by the use of a column heater and by insulating the heater in order to prevent any changes in temperature. Baseline drift also caused considerable interference in the chromatograms, this was largely as a result of the very low UV wavelength that was used for detection purposes. Interference from the acetonitrile in the mobile phase contributed to this drift, the problem

was abolished when acetonitrile from a different manufacturer was used and by continuously degassing the mobile phase with helium during the analytical run.

The extraction conditions of the fatty acids were investigated in order to optimize the conditions of the sample preparation and improve the reproducibility of the assay. The time of 10 minutes was chosen, even though the recoveries of three fatty acids were beginning to decrease, because the smallest variation in the results of arachidonic, linoleic, linolenic and palmitoleic acids occurred at this time. The effect of time on the hydrolysis and release of free fatty acids at 70°C showed that maximum recovery of the fatty acids occurred after 35 minutes, in all fatty acids except for palmitic acid. However, as this fatty acid was not one of crucial importance, the experiment was not repeated and 35 minutes was used as the heating time for all further work.

The main drawback was the poor reproducibility of the saturated fatty acids, palmitic, stearic and also oleic acid. This was due to the lack of a chromophore in the molecule, which would have enabled increased sensitivity during spectrophotometric detection. However, the detector response for the fatty acids of main interest, namely linoleic acid and its conjugated diene, was higher due to the presence of the unsaturated bonds enabling satisfactory reproducibility of these fatty acids.

In order to enhance the sensitivity of oleic, palmitic and stearic acid a derivatization method labelling the fatty acids with 4-bromomethyl 7-methoxy coumarin, using crown ether as a catalyst, was attempted (Lam and Grushka 1978; Jüngling and Kammermeier 1988). Enhanced sensitivity using fluorescence detection was achieved, however, it became very difficult to identify the fatty acids, especially the conjugated diene isomer of linoleic acid and so further work on the method was discontinued.

In conclusion, the quantitative determination of the conjugated diene (9-*cis*, 11-*trans*-octadecadienoic acid) and its molar ratio to linoleic acid was achieved by this method with satisfactory reproducibility.

4. Measurement of Ascorbic Acid and Dehydroascorbic Acid in Plasma by HPLC

4.1 Introduction

In this study, an isocratic HPLC method using the mobile phase of De Antonis *et al.* (1993) was developed for the direct measurement of AA and the indirect determination of DHAA in plasma. Electrochemical detection was employed for the measurement of AA as this form of detection was more selective and offered greater sensitivity than UV detection. Ascorbic acid is easily oxidized and electrochemically active, DHAA is not electrochemically active and its determination was therefore indirect. Each sample was analysed twice, once for the AA content and again after the reduction of DHAA to AA by dithiothreitol, to give the total AA content (AA+DHAA). The DHAA concentration was then calculated as the difference between the two values.

4.2 Equipment

The HPLC instrumentation consisted of a series 410 LC pump, an ISS-100 autosampler, an LC-4B amperometric detector and an LCI-100 laboratory computing integrator (Perkin-Elmer Ltd., Buckinghamshire, UK). The separation was performed on a Spherisorb 5 μm ODS-1 analytical column, 25 cm x 0.46 cm, with a 5 cm guard column (Jones Chromatography, Hengoed, Mid-Glamorgan, UK). The guard column contained 10 μm ODS and was repacked after 50-100 sample injections. A 20 μl injection volume was used and the optimal potential of the working electrode was found to be +0.725 V. Capped polypropylene centrifuge tubes (2 ml) were used for the sample preparation (BDH, Merck Ltd., Poole, Dorset, UK).

4.3 Chemicals and Reagents

L-Ascorbic acid, EDTA, metaphosphoric acid (MPA), sodium acetate and glacial acetic acid (all A.R. grade) were obtained from BDH, Merck Ltd., Poole, Dorset, UK. L-Dehydroascorbic acid, dithiothreitol (DTT), 3,4 dihydroxybenzylamine hydrobromide (DHBA) and sodium octane sulfonate sodium salt were obtained from Sigma Chemical Co.,

Dorset, UK. Methanol (HPLC grade) was purchased from Rathburn Chemicals Ltd., Walkerburn, Scotland.

All solutions were prepared with de-ionized water. A 10% solution of MPA, freshly prepared, was used to precipitate the plasma proteins. A 10 mmol/l DTT solution, for the reduction of DHAA to AA, was prepared and stored at 4°C for one up to one month.

Internal standard solutions

A stock solution of internal standard, DHBA (2 mmol/l), in hydrochloric acid (10 mmol/l), was prepared and stored at 4°C. A working internal standard solution (80 μmol/l) was prepared by diluting 1 ml of the stock DHBA solution to 25 ml with 10% MPA. The final concentration of the internal standard in the samples, after the sample preparation had been completed, was 40 μmol/l.

Ascorbic acid standard solutions

A 500 μmol/l stock solution of AA, previously dried to constant weight over silica gel in a vacuum desiccator, was prepared in 3% MPA. Working standard solutions with concentrations of 10, 20 and 30 μmol/l were prepared by diluting 1, 2 and 3 ml of the stock solution to 50 ml with 3% MPA respectively. Each solution also contained 1 ml of the stock internal standard, DHBA, with a resulting concentration of 40 μmol/l. All solutions were prepared immediately before use and were protected from light at all times. A 20 μl volume of each standard was injected on to the column in order to obtain a standard curve for the analysis.

Mobile phase

The mobile phase was prepared according to the method of De Antonis et al. (1993) by dissolving 6.56 g of sodium acetate, 0.216 g of sodium octane sulfonate, 0.034 g of EDTA and 52.6 ml of methanol in 850 ml of water. The pH was adjusted to 4.0 with glacial acetic acid and the final volume made up to 1l. The mobile phase was filtered, sparged with helium prior to use and delivered isocratically with a flow rate of 1.2 ml/minute.

4.4   Sample Preparation

Ascorbic acid

The AA content of plasma was determined by placing a 200 µl aliquot of plasma into a 2 ml centrifuge tube, 200 µl of water and 400 µl of the working internal standard solution in 10% MPA were then added. The samples were mixed gently by inversion to facilitate protein precipitation and then frozen at -70°C. At the time of analysis, the samples were thawed for 5 minutes at room temperature, centrifuged at 2500 x *g* for 2 minutes and 20 µl of the clear supernatant was injected on to the column for analysis.

Dehydroascorbic acid

The total AA content of the sample (DHAA+AA) was determined by reducing the DHAA back to AA with DTT. To 200 µl of plasma, 200 µl of DTT (10 mmol/l) were added and the reduction of DHAA allowed to proceed for 6 minutes at room temperature, before the addition of 400 µl of the working internal standard in 10% MPA. The samples were mixed and then frozen at -70°C. At the time of analysis the samples were thawed for 5 minutes, centrifuged and 20 µl of the clear supernatant analysed. The DHAA concentration was then calculated as the difference between the total AA content (DHAA+AA) and the AA content.

4.5   Method Validation

Detector response

The optimum potential of the working electrode was found by preparing a standard solution of AA (20 µmol/l) and repeating the analysis over a range of detector potentials (+0.5 to +0.95 V). The detector was then set at the potential with the maximum response to the analyte concentration. The linearity of the detector response was then evaluated at the optimum potential by preparing and analysing a series of AA standards ranging from 5 to 80 µmol/l, each with an internal standard concentration of 40 µmol/l. By further diluting the standard with the lowest concentration, the minimum detectable amount was obtained.

Reduction of dehydroascorbic acid to ascorbic acid

In order to optimize the conditions for the determination of total AA, the effects of the DTT

concentration and the reaction time on the reduction of DHAA to AA at room temperature were investigated. Dithiothreitol solutions with concentrations of 5 and 10 mmol/l were prepared. Fresh plasma was obtained and divided into two sets, one for each DTT concentration. Each set was divided into aliquots; DTT was added to each aliquot and the reaction allowed to proceed at room temperature in the dark. At intervals of two minutes, the working internal standard solution in 10% MPA was added to successive samples in each set in order to stop the reaction and stabilize the AA for analysis.

Precision

A fresh plasma sample was obtained from a healthy volunteer. Samples were prepared for the analysis of AA and DHAA and then frozen and stored at -70°C. The within batch precision of the method was determined by thawing and analysing samples on the same day under identical conditions. The remaining samples were stored and one sample was included in each analytical run enabling the between batch variation to be calculated.

Accuracy

The AA concentration of a plasma sample was determined with and without the addition of standard AA solutions of either high (30 μmol/l) or low (8 μmol/l) molarity. Quadruple sets of samples were analysed and the percentage of each standard recovered was calculated.

Stability

The stability of AA, in whole blood and plasma, prior to protein precipitation and stabilization in acidic conditions was investigated. Blood from a healthy individual was collected into 4 ml EDTA Vacutainer tubes (Becton Dickinson Ltd., Oxford) ($n = 14$). The plasma from one tube was immediately separated and analysed for AA and DHAA content. The remaining tubes were divided into two sets: one set was placed in a refrigerator at 4°C and the other was kept at room temperature (25°C), both sets were protected from the light. At intervals of 1, 2, 3, 4 and 6 hours, one tube of whole blood was removed from each set, the plasma was separated and immediately analysed for AA and DHAA content.

To assess the stability of ascorbic acid in plasma prior to acidification, a fresh plasma sample was obtained and immediately analysed for the content of AA and DHAA. The remainder of the plasma was divided into two aliquots, one was kept at 4°C and the other at room temperature (25°C), both were protected from light and analysed for AA and DHAA content over a 6 hour period. To establish the effect of long term storage, a fresh plasma sample was obtained and immediately analysed for the AA content. The remainder was stored at -70°C and analysed on a monthly basis.

4.6   Results

Detector response

The optimum potential for the analysis of AA was found to be +0.725 V and the detector was set at this potential. The standard curve for AA is shown in Figure 4.1. The peak height ratios of the AA standards to the internal standards were linear to at least 80 μmol/l. The minimum detectable amount was the amount of analyte present in a 20 μl injection volume giving a peak whose height was twice that of the baseline noise; this was found to be 74 nmol/l.
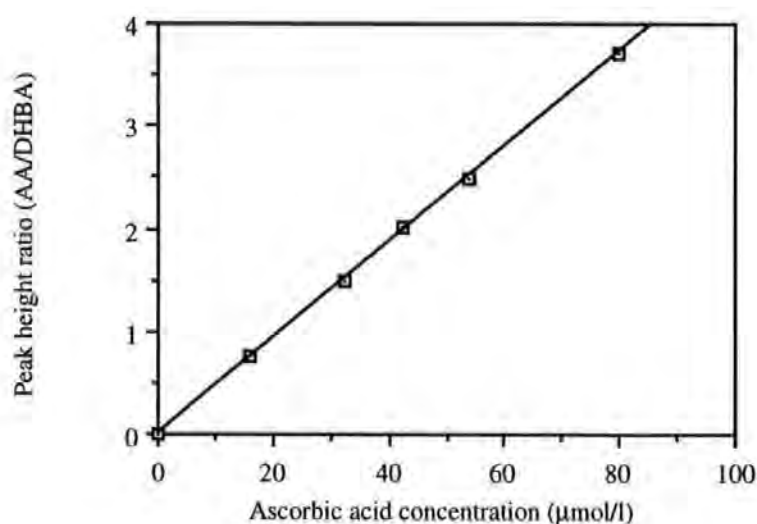
Figure 4.1 *Standard curve for the peak height ratios of ascorbic acid / internal standard (DHBA) against concentration.*

Typical chromatograms of a standard solution and a plasma sample are shown in Figures 4.2 and 4.3. The order of elution was established by analysing each component separately and noting the retention time. The AA peak in a plasma sample was identified from the retention time and by 'spiking' the sample with a standard solution of AA. Additionally, plasma depleted of AA, by storage at 4°C for 24 hours prior to acid stabilization was also analysed. The absence of the AA peak gave further confirmation of the peak identity and also indicated that AA was not co-eluting with any other compound. Baseline resolution between AA, uric acid, DTT and DHBA was achieved and the analysis was completed within 12.5 minutes.

Reduction of dehydroascorbic acid to ascorbic acid

During the investigation of the reduction of DHAA to AA in plasma, the effects of 5 and 10 mmol/l DTT were examined (Figure 4.4). The DTT solutions were added to plasma in a 50:50 ratio, so that the final concentrations were 2.5 and 5 mmol/l respectively. The reduction of DHAA appeared to reach a maximum between 6 and 8 minutes using 5 mmol/l DTT. A higher result was obtained with 10 mmol/l DTT and the reaction reached a maximum between 4 and 6 minutes. On the basis of these results, a reaction time of 6 minutes using 10 mmol/l DTT was chosen for the reduction of DHAA at room temperature, before stabilizing the samples in acid and freezing at -70°C.
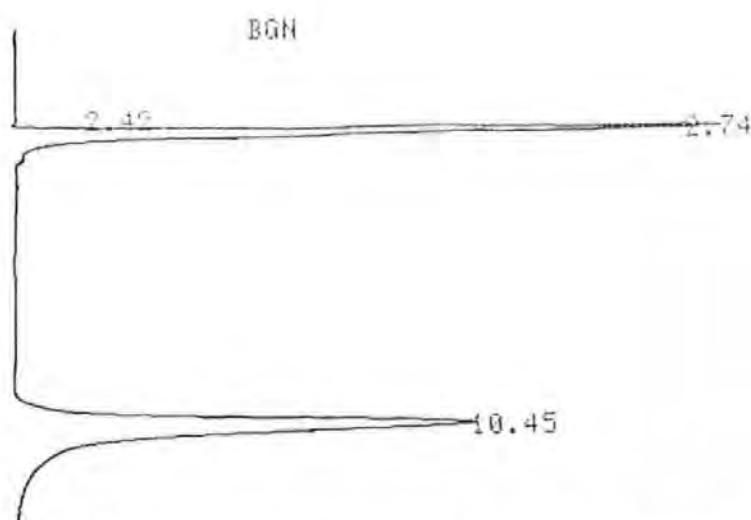
Figure 4.2. *A typical chromatogram of a standard solution of AA (retention time 2.74 min) and internal standard DHBA (retention time 10.45 min).*
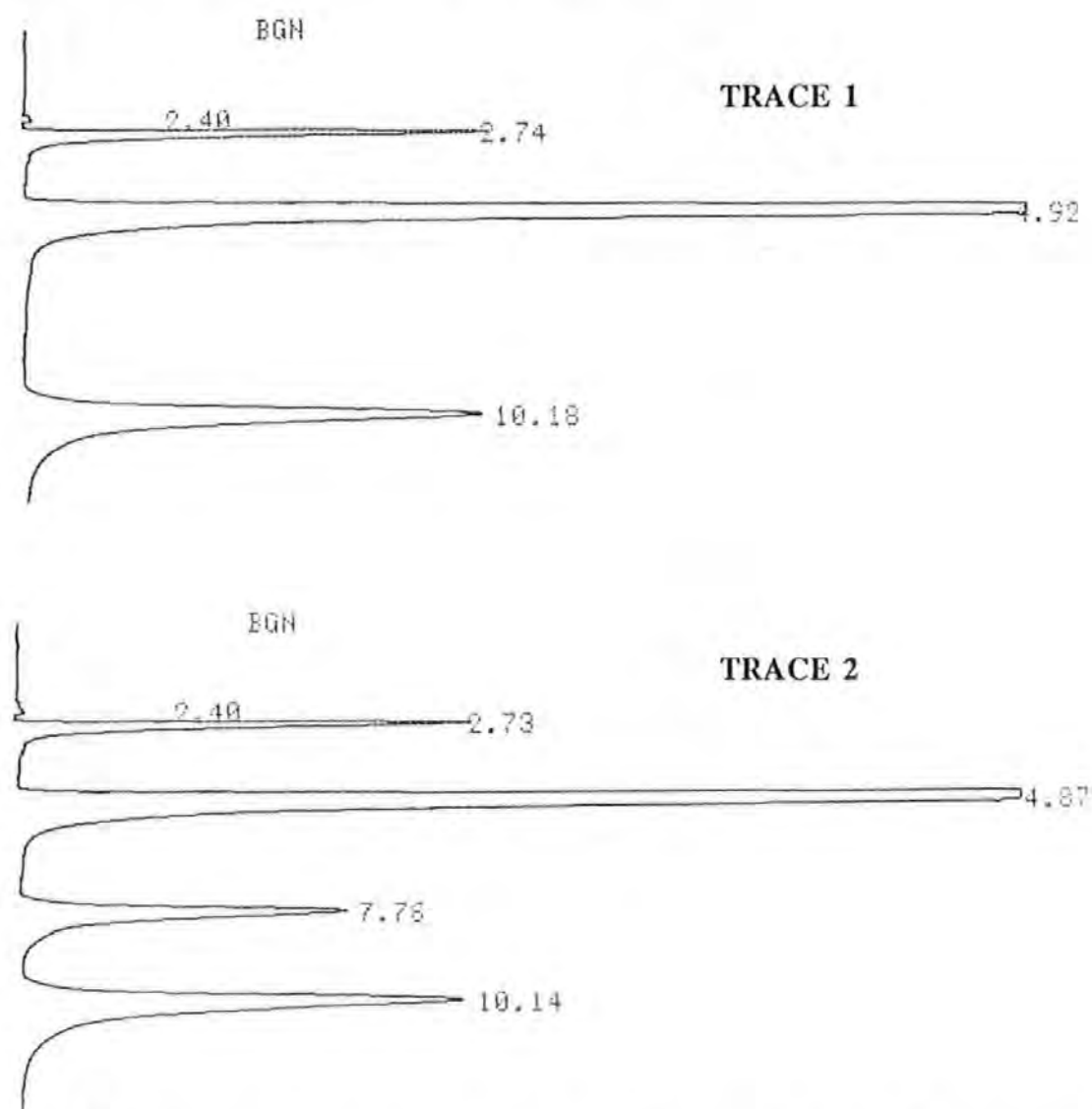
Figure 4.3 *A typical chromatogram of a plasma sample. Trace 1 showing the separation of AA (2.74 min) from uric acid (4.92 min) and DHBA (10.18 min); Trace 2 (total AA) showing the separation of AA (2.73 min) from uric acid (4.87 min), DTT (7.76 min) and DHBA (10.14 min).*

Precision

The within batch coefficient of variation was 5.5 % ($n = 6$) for AA and 7.6% ($n = 6$) for DHAA. The between batch coefficient of variation was 6% ($n = 5$) for AA and 8% ($n = 5$) for DHAA .

Accuracy

The addition of 8 and 30 μmol/l of AA to plasma, gave mean recoveries of 91.1% ($n = 4$) and 95.6% ($n = 4$) respectively.

Stability

The concentration of AA in two sets of plasma (1 and 2) stored at -70°C for 6 months is shown in Figure 4.5. No decrease in the concentration of AA was observed in set 1. A slight decrease, however, was observed in set 2, although this was within the variability of the method.

The stability of AA in whole blood prior to the separation of plasma and addition of 10% MPA is shown in Figure 4.6. In whole blood stored at 4°C, a decrease of 6% in the total AA content was observed after 2 hours. After 6 hours, 66% of the AA had been lost. A more rapid decline in the concentrations of AA were observed in whole blood samples kept at 25°C, with a 13% decrease after 2 hours and a decrease of 80% after only 4 hours. In plasma stored at 4°C and 25°C prior to the addition of MPA, the decline in AA content was more pronounced over the first hour of storage (Figure 4.7). After 2 hours, 27% of the AA had been lost from plasma kept at 4°C and 43% from plasma kept at 25°C. After 6 hours at 4°C, 41% of the AA had been lost and at 25°C the decrease in AA approached 80%. In view of these results, plasma was separated and stabilized in acid, within 2 hours of the collection of blood samples.

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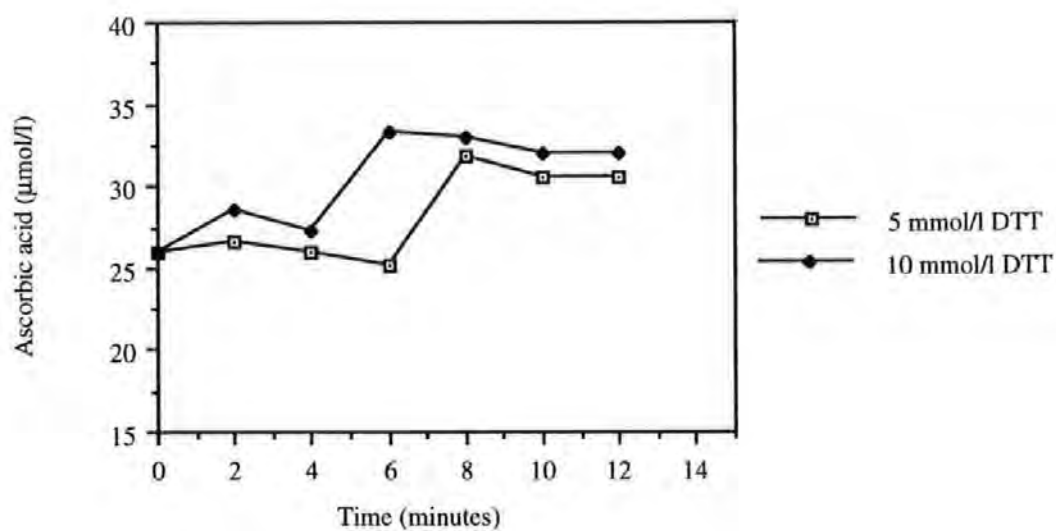
Figure 4.4 *The effect of 5 and 10 mmol/l DTT on the reduction of DHAA to AA in plasma at room temperature.*
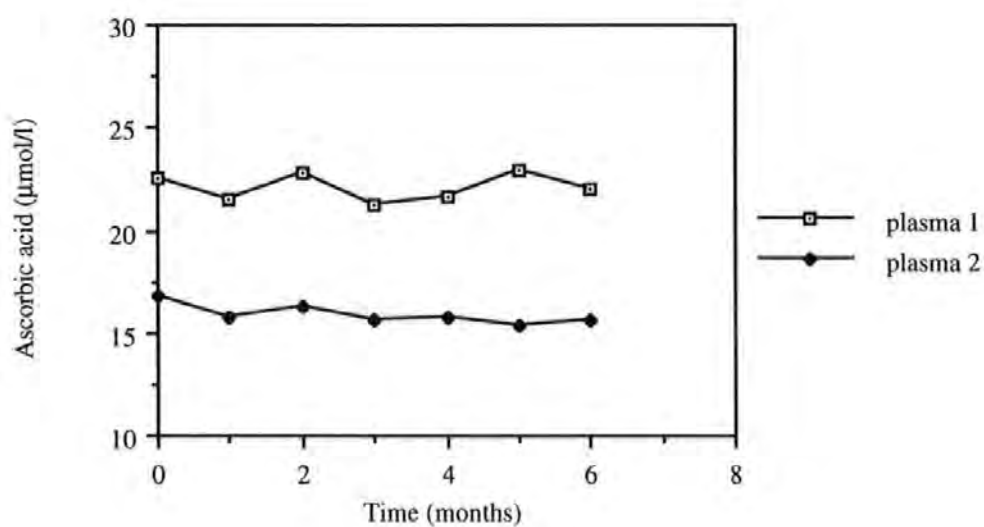
Figure 4.5 *Ascorbic acid concentration in two sets of plasma stored at -70°C.*
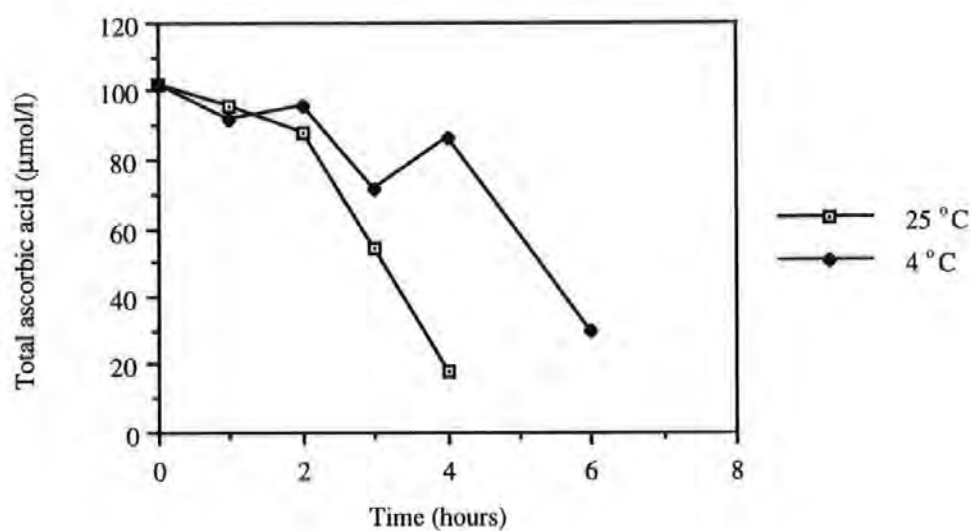
Figure 4.6 *The stability of AA in whole blood samples stored at 4°C and 25°C prior to the preparation of plasma samples.*
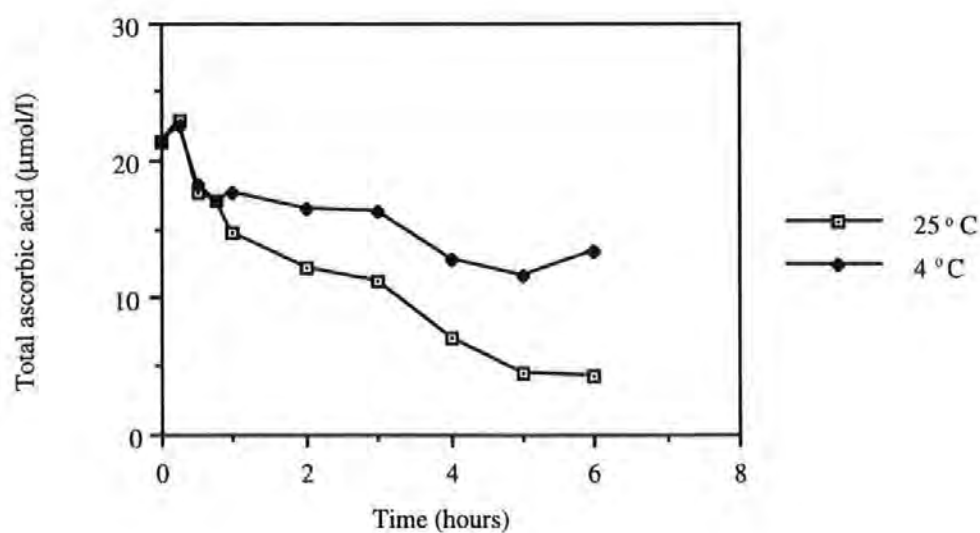
Figure 4.7 *The stability of AA in plasma stored at 4°C and 25°C prior to acid stabilization.*

4.7   Discussion

A plethora of HPLC methods exist for the determination of AA (reviewed by Washko *et al.* 1992 and Rizzolo and Polesello 1992). The most common forms of HPLC are reverse phase, reverse phase with ion-pairing agents and weak anion-exchange with amino bonded phases. Problems still remain in current methods: difficulties arise in separating AA from other water-soluble components, especially uric acid in plasma samples and there are no single step HPLC assays available for the simultaneous detection of a AA and DHAA.

During the initial investigations of HPLC methods for the determination of AA, several methods were tried. The reverse-phase method of Barja de Quiroga *et al.* (1991) with UV detection was attempted. This produced an excellent separation of AA from uric acid in standard solutions, but the analysis of plasma samples proved problematic. The injection of approximately ten successive plasma samples resulted in large changes in retention times and the elution of a retained compound which caused further interference in the assay. These problems were caused by the precipitation of the ion-pairing agent (tetradecyl trimethyl ammonium bromide), in the mobile phase, by the acid in the samples. Similar problems were encountered by Liau *et al.* (1993) using octylamine as an ion-pairing agent.

Several attempts were made to resolve these problems. Different acids were tried for the sample preparation, however, the most commonly used acids, metaphosphoric acid, perchloric acid and trichloroacetic acid all caused precipitation. Dilution of the plasma samples prior to injection in order to reduce the ionic strength of the acid resulted in decreased sensitivity and reproducibility of the assay and highlighted the need for an internal standard. Reducing the amount of the ion-pairing agent in the mobile phase from 1 to 0.3 mmol/l enabled the resolution of the assay to be maintained, but did not resolve the problem of precipitation. Different ion-pairing agents were tried, but the loss of resolution then became a problem and precipitation occurred when ion-pairing agents with a similar structure to tetradecyl trimethyl ammonium bromide were used.

The method of Hatch and Sevanian (1984) using a weak anion-exchange column for the separation of AA with UV detection was also attempted. Difficulties arose in achieving a good separation of AA from uric acid with this method and in reproducing the chromatographic conditions on a daily basis. A within batch precision of 7% and a between batch precision of 15% were obtained. Precipitation also occurred upon the addition of acetonitrile to the phosphate buffer during the preparation of the mobile phase, creating another, albeit minor, problem with this method. The analytical run times approached 25 minutes and peak broadening occurred.

The HPLC method of De Antonis *et al.* (1993) using electrochemical detection for the determination of vitamin C was also attempted. The use of electrochemical detection resulted in increased sensitivity and no problems were encountered with plasma samples prepared with 10% MPA. An excellent separation of AA from uric acid was produced, and the analysis of plasma depleted of AA produced the finding that the AA peak was not contaminated by any other compound. Hence, this method was used for all further work.

The detector response was linear up to at least 80 μmol/l and encompassed the AA concentration range expected in clinical samples.

Dihydroxybenzylamine hydrobromide was found to be a suitable internal standard, without causing any interference in the detection of AA. This proved advantageous in terms of the reproducibility of the assay. Other workers used tyrosine as an internal standard (Doner and Hicks 1981) or isoascorbic acid, a stereoisomer of AA (Lopez-Anaya and Mayersohn 1987; Koh *et al.* 1993).

High background currents and electrode poisoning have been reported with the use of electrochemical detection (Ziegler *et al.* 1987; Wagner and McGarrity 1991). These problems were not encountered in this study. The electrode surfaces were cleaned with deionized water after each analytical run and the reference electrodes were stored in 3 mol/l sodium chloride whilst not in use.

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Okamura (1980) described a spectrophotometric method for the determination of AA and DHAA in which DHAA was reduced to AA by a suitable reducing agent. The reaction conditions were investigated and the most suitable reducing agent was found to be DTT. The conditions for the reduction of DHAA to AA with DTT were dependent upon the pH, the DTT concentration and time. The optimum pH was found to be between 6.5 to 8.0 (Okamura 1980; Ødum 1993). Okamura (1980) also reported that a DTT concentration of 2.5 mmol/l was sufficient for the reduction of 570 μmol/l of DHAA in plasma, with a reaction time of 10 minutes at room temperature, without the requirement of a buffer solution to maintain the pH of the reaction.

Since DHAA is electrochemically inactive, direct determination was not possible and DHAA was measured indirectly by reduction to AA using DTT as described above. When the DTT concentration and reaction time were investigated, it appeared that the reaction was not complete at DTT concentrations below 5 mmol/l, as depicted in Figure 4.4. The stability results showed a rapid decline of AA in plasma not stabilized in acid conditions, therefore, the minimum reaction time for the reduction of DHAA to AA was the preferred option. A maximum reduction of DHAA was obtained when 10 mmol/l DTT with a reaction time of 6 minutes were used at physiological pH (7.4). Room temperature was chosen as the reaction temperature for convenience. This procedure allowed the total AA content of plasma to be measured and enabled the DHAA content to be determined indirectly. Although the disadvantage with this method was that a small increase was being measured over a large background, the procedure required little additional sample manipulation and electrochemical detection provided the sensitivity necessary to measure the increase.

Direct measurement of DHAA by HPLC, with UV detection at 210 nm, was achieved by Rose and Nahrwold (1981). Cammack *et al.* (1991) also used UV detection for the direct measurement of DHAA, whilst simultaneously measuring AA with electrochemical detection. However, this form of direct determination lacks sensitivity due to the low UV absorptivity of DHAA and is subject to interference at the low wavelengths used for detection. Keating and Haddad (1982) and Speek *et al.* (1984) were successful in

enhancing the sensitivity of DHAA by precolumn derivatization with 1,2-phenylenediamine, although problems with the stability of the derivative were experienced.

The accurate determination of DHAA is difficult due to its instability and this is reflected by the discrepancies that exist in the literature concerning the levels found in plasma. The DHAA concentration in plasma (mean ± SD) is reported to be: 1.44 ± 1.67 μmol/l ($n = 10$) (Okamura 1980), 12.0 ± 3.7 μmol/l ($n = 20$) (Lunec and Blake 1985), 5.8 ± 2.7 μmol/l ($n = 15$) (Nagy and Degrell 1989), see also Table 1.6 (page 62). A high concentration (>20 μmol/l) of DHAA has been reported in diseased states: rheumatoid arthritis and diabetes (Lunec and Blake 1985; Jennings *et al.* 1987b; Banerjee 1982). Unfortunately, the optimum conditions for the determination of DHAA were developed after the collection of samples for the weight loss study had begun. Thus, in these samples only the ascorbic acid content could be determined. However, during the method validation DHAA was measured in healthy subjects and the concentration was found to be 12.8 ± 8.7 μmol/l (mean ± SD) ($n = 6$) and in diabetic patients 2.72 ± 2.19 μmol/l ($n = 29$).

There is a lack of published data regarding the stability of AA during sample collection and long term storage. In routine laboratory procedures, several hours can elapse between the collection of blood samples and sample preparation. Hence in this study, the stability of AA in whole blood and plasma prior to acid stabilization was examined, in addition to the effect of long term storage of samples at -70°C.

The results from this study showed a rapid decline in AA content in whole blood after 2 hours of storage at 4°C and 25°C. Similar results were observed for plasma with a greater initial loss in AA occurring during the first hour. These results were in contrast to those of Liau *et al.* (1993) who showed that AA was stable in whole blood for 4 hours prior to the deproteinization of plasma. Schorah *et al.* (1996) stored whole blood from critically ill patients and healthy controls for 4 hours at room temperature prior to the separation of plasma and stabilization in MPA. The loss in AA was significantly greater in the critically ill group after 4 hours when compared to the control group. The results from these studies

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suggest that inter-individual differences in the stability of AA in whole blood may occur and may be dependent upon the initial concentration of AA or on the levels of other plasma components. A recent study has found that AA is stable for 6 hours at 4°C in blood samples collected in trisodium citrate for the preparation of plasma, whilst in blood samples collected in plain tubes for the preparation of serum, AA was stable for 24 hours (Key *et al.* 1996). These results indicate that the stability of AA is also affected by the conditions used for the collection of blood samples. In this study, blood samples were collected in tubes containing EDTA and precautions were taken to separate the plasma and stabilize the AA in MPA within 2 hours of the samples being taken.

Variability concerning the long term storage of AA in plasma stabilized in acidic conditions is also found in the literature. These differences may result from the use of different stabilizing media. In summary, AA appeared to be stable for 24 hours at 4°C in 5% MPA (Margolis *et al.* 1990) and methanol containing trichloroacetic acid (Moeslinger *et al.* 1995), or for 12 hours at 5°C in 10% MPA (Ødum 1993). At -20°C the AA concentration in plasma preserved with 10% MPA was found to be stable for 2 weeks (Ødum 1993) and 3-4 weeks (Lunec and Blake 1985; Moeslinger *et al.* 1995). Nagy and Degrell (1989) reported that AA could be stored for 22 days at -34°C, without decrease, in plasma preserved with 0.2 mmol/l MPA and 5 mmol/l EDTA. Margolis and Davis (1988) stored plasma supplemented with AA and preserved with DTT and MPA (5%) for extended periods. The results showed that AA remained stable for at least 57 weeks at -70°C. The study was extended to cover a period of 6 years. Plasma preserved with DTT and MPA showed a decrease in AA of approximately 4-7% per year of storage; plasma freeze-dried before storage at -70°C was found to be stable for 6 years (Margolis and Duewer 1996). In this study, AA was found to be stable for at least 6 months in plasma preserved with 10% MPA at -70°C.

Ascorbic acid does not appear to be stable at high temperatures. In plasma preserved with 5% MPA, detectable oxidation of AA had occurred after 5 hours at 25°C (Margolis *et al.* 1990). Thus, as a further precaution, samples were removed from the freezer, thawed and immediately injected on to the column at the time of analysis in order to minimize losses.

In conclusion, the methodology described, offers straight-forward sample preparation for the determination of AA and DHAA in plasma, with excellent separation of AA from uric acid, DTT and internal standard, with a short analysis time. The stability data indicated that the rapid separation of plasma and stabilization in acidic conditions is of crucial importance in the study of AA status in human subjects.

5. Measurement of Retinol and α-Tocopherol in Plasma by HPLC

5.1 Introduction

High performance liquid chromatography has enabled straight forward, sensitive, rapid and accurate analysis of vitamin A (all-*trans* retinol) and vitamin E (α-tocopherol) in biological samples. An isocratic, reverse phase method, for the simultaneous determination of retinol and α-tocopherol with UV detection was used in this study (MacCrehan and Schönberger 1987). The preparation of plasma samples involved three stages: denaturation of the lipid-protein associations using absolute ethanol, extraction of the vitamins using an organic solvent and solvent exchange, evaporating the extracting solvent and reconstituting the vitamins in suitable medium for direct analysis.

5.2 Equipment

The HPLC equipment consisted of a Model 501 pump (Millipore, Waters, Hertfordshire, UK), a Model 9050 variable wavelength UV/VIS detector (Varian Associates Ltd., Surrey, UK) and a Model 3395 integrator (Hewlett-Packard Ltd., Stockport, Cheshire UK). For sample injection, a Model 7010 manual injector (Rheodyne, Macclesfield, Cheshire, UK), equipped with a 20 µl loop was used. Chromatographic separation was performed on a Spherisorb 5 µm ODS-2 column, 25 cm x 0.46 cm, with a 5 cm guard column containing 10 µm ODS purchased from Jones Chromatography, Hengoed, Mid-Glamorgan, UK. Other equipment included a PU 8625 series UV/VIS spectrophotometer (Philips Analytical, Cambridge, UK).

5.3 Chemicals and Reagents

All-*trans* retinol, retinol acetate, α-tocopherol and tocopherol acetate were obtained from Sigma Chemical Co., Dorset, UK. Ascorbic acid (A.R.) methanol (HPLC grade), ethanol (GPR) butan-1-ol (GPR), and hexane (A.R.) were obtained from BDH, Merck Ltd., Dorset, UK.

Standard solutions

Stock standard solutions of retinol (28 μmol/l) and α-tocopherol (232 μmol/l), were prepared by dissolving approximately 2 mg of retinol in 250 ml of ethanol and 10 mg of α-tocopherol in 100 ml of ethanol. The exact concentrations of the solutions were determined spectrophotometrically, by using the extinction coefficients (E1% 1 cm) in ethanol. The values used at the wavelength maxima were: retinol 1850 at 324 nm and α-tocopherol 75.8 at 292 nm (Kaplan *et al.* 1987; MacCrehan and Schönberger 1987; Catignani and Bieri 1983).

Stock solutions of the internal standards, retinol acetate (21 μmol/l) and tocopherol acetate (1057 μmol/l), were prepared by dissolving 0.7 mg of retinol acetate in 100 ml of ethanol and 50 mg of tocopherol acetate in 100 ml of ethanol. All solutions were protected from light and stored at 4°C for up to one month.

A combined working standard solution containing retinol (2.8 μmol/l), α-tocopherol (23.2 μmol/l), retinol acetate (2.1 μmol/l) and tocopherol acetate (105.7 μmol/l) was prepared by diluting 5 ml of each stock standard solution to 50 ml, with ethanol containing AA (1 g/l) as an antioxidant. A 20 μl sample of this working standard solution was directly injected on to the column for analysis.

A combined solution of the internal standards, retinol acetate (2.1 μmol/l) and tocopherol acetate (105.7 μmol/l), was prepared by diluting 5 ml of the stock internal standard solutions to 50 ml with ethanol containing AA (1 g/l). This solution was used for the sample preparation.

Mobile phase

The mobile phase consisted of water / methanol / butan-1-ol in the ratio 2.5 / 87.5 / 10 by volume. The mobile phase was degassed with helium for 5 minutes before use and then delivered isocratically with a flow rate of 1.5 ml/minute.

5.4 Sample Preparation

All procedures were performed in subdued daylight at room temperature. Frozen plasma was thawed and mixed gently to resuspend any material. A 200 µl aliquot of plasma was transferred to a glass centrifuge tube and 200 µl of the combined internal standard solution were added. The sample was vortex mixed for 15 seconds and 400 µl of hexane were added. After mixing for 30 seconds the sample was centrifuged at 1000 x g for 2 minutes. The hexane layer was transferred to another glass centrifuge tube and the extraction process with hexane repeated twice more. The combined hexane extracts were evaporated to dryness under a constant stream of nitrogen and the residue immediately reconstituted in 200 µl of ethanol. A 20 µl portion was then directly injected on to the column for analysis.

5.5 Method Validation

Detector Response

The UV detector was programmed to switch wavelengths from 324 nm to 292 nm after 5.15 minutes, enabling both retinol and α-tocopherol to be measured at their maximum wavelengths. The linearity of the detector response was evaluated by preparing and analysing a series of retinol and α-tocopherol standards, with each solution containing an identical concentration of the corresponding internal standard. Standard curves were obtained by plotting the peak height ratios for each analyte to its corresponding internal standard, against the analyte concentration. By further diluting the standard with the lowest concentration, the minimum detectable amount was obtained.

Precision

The within batch variation of the method was determined by obtaining a fresh plasma sample and repeating the analysis on the same day under identical conditions. The remainder of the plasma was frozen in 250 µl aliquots at -70°C. Each analytical run included one aliquot which enabled the between batch variation to be calculated.

Accuracy

The retinol and α-tocopherol concentrations of a plasma sample were determined with and without the addition of standard solutions. The volumes of the added solutions were kept low, 10 μl, so as not to cause protein precipitation and the percentage of the standard recovered from the plasma was calculated.

Stability

A plasma sample was obtained from a healthy volunteer and analysed without delay. The remainder was separated into 250 μl aliquots and stored at -70°C and -20°C. Samples were analysed on a monthly basis.

5.6 Results

Figure 5.1 shows a typical chromatogram of the combined working standard solution. All standards are clearly resolved from the internal standards and eluted by 11 minutes. The order of elution was established by running each standard singly and noting the retention time. A chromatogram of a plasma sample is shown in Figure 5.2. The peaks were identified by their retention times; further confirmation of peak identity was achieved by 'spiking the samples with small amounts of standard solutions.

Figures 5.3 and 5.4 show the standard curves for retinol and α-tocopherol. The peak height ratios of the standards to the internal standards were linear to concentrations of at least 12.5 μmol/l for retinol and 220 μmol/l for α-tocopherol. The slope of the each graph gave a constant, the response factor; by dividing the peak height ratio of the sample by the response factor the concentration of the analyte in a plasma sample was calculated.

The detection limit was the amount of analyte present in a 20 μl injection volume giving a peak whose height was twice that of the baseline noise. This was found to be 33 nmol/l for retinol and 0.96 μmol/l for α-tocopherol.
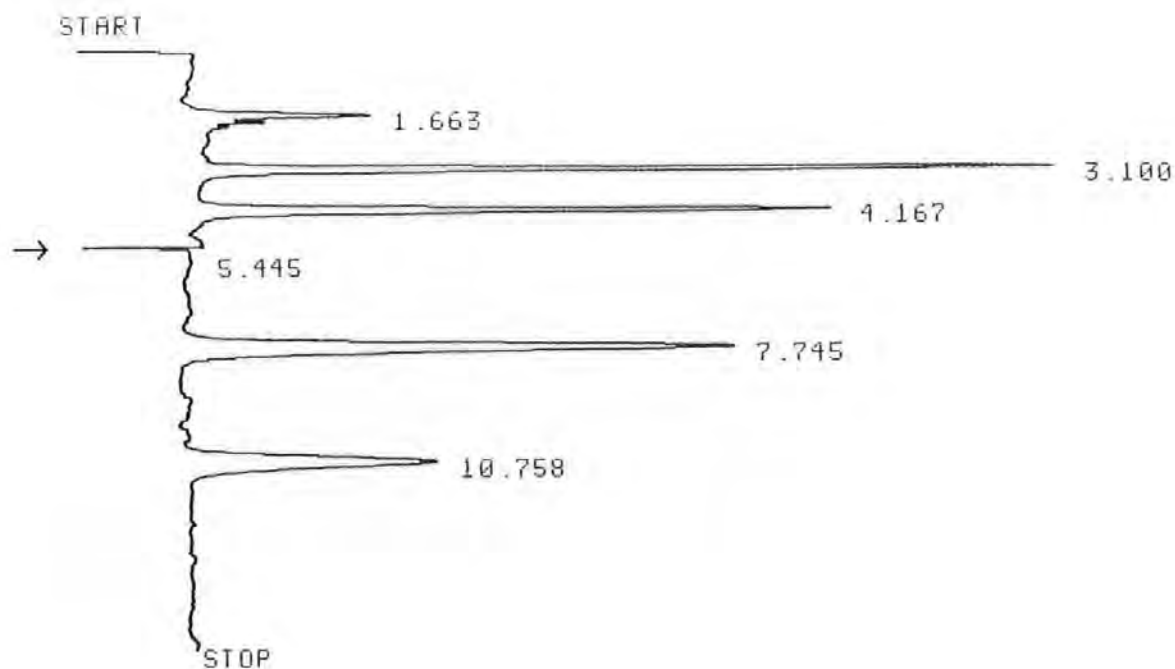
Figure 5.1 *A typical chromatogram of a standard mixture of retinol (retention time 3.10 min), retinol acetate (4.16 min), α-tocopherol (7.74 min) and tocopherol acetate (10.75 min). The detector response was set at 0.05 AUFS. The arrow indicates the change in wavelength from 324 nm to 292 nm.*
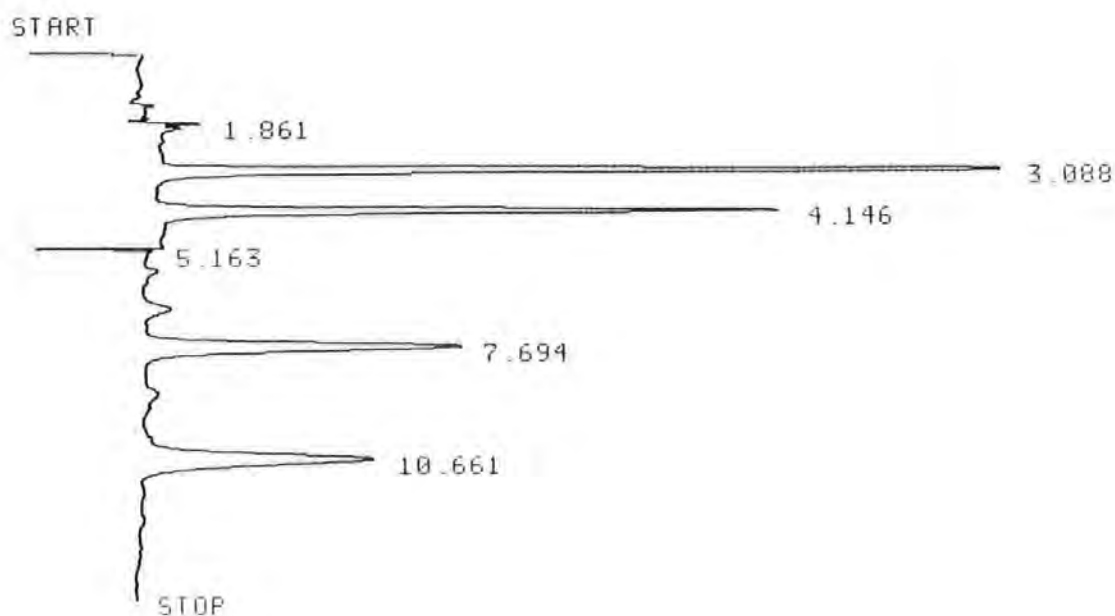
Figure 5.2 *A typical chromatogram of a plasma sample showing retinol (3.08 min), retinol acetate (4.14 min), α-tocopherol (7.69 min) and tocopherol acetate (10.66 min).*
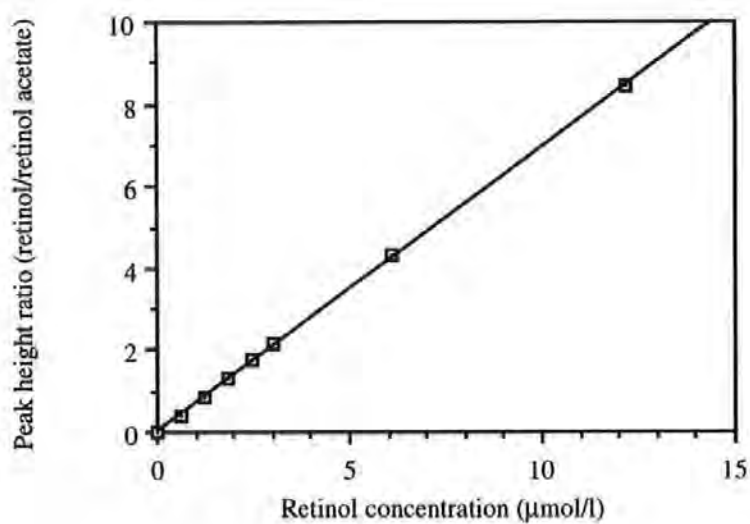
Figure 5.3 *Standard curve for the peak height ratios of retinol / retinol acetate against concentration.*
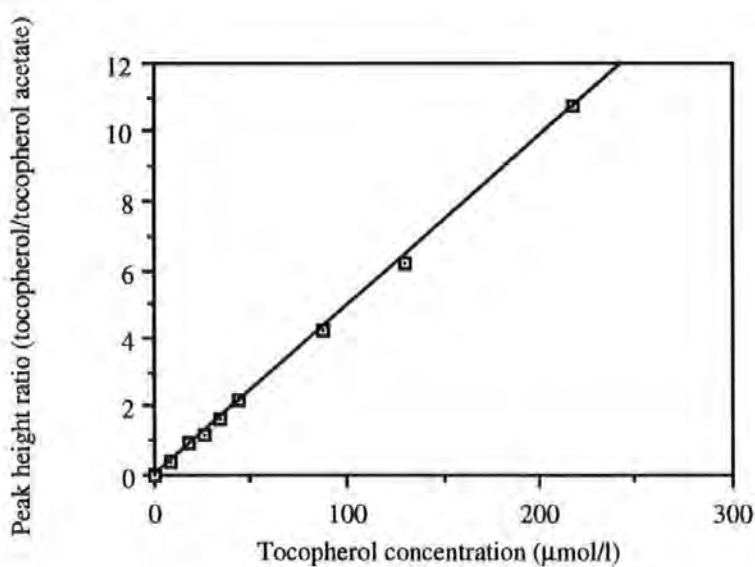
Figure 5.4 *Standard curve for the peak height ratios of α-tocopherol / tocopherol acetate against concentration.*

Precision

The within batch variation of the method, expressed as the coefficient of variation, was 1% (n = 10) for retinol and 3% (n = 10) for α-tocopherol. The between batch variation was 3% (n = 8) for retinol and 5% (n = 8) for α-tocopherol.

Accuracy

The addition of 1.5 μmol/l of retinol to plasma, of known retinol concentration, gave a mean recovery value of 94.6% (n = 4) and the addition of 5 μmol/l of α-tocopherol resulted in a mean recovery of 93% (n = 4).

Stability

The results of 12 months of storage of plasma are shown in Figures 5.5 and 5.6. No decrease in the concentrations of retinol or α-tocopherol were observed in plasma stored at -70°C. However, a decrease in both the retinol and α-tocopherol concentrations, greater than the between batch variation of the method, were observed after 9 months of storage at -20°C. After 12 months of storage at -20°C the concentration of retinol had decreased by 9% and the concentration of α-tocopherol had decreased by 22%.
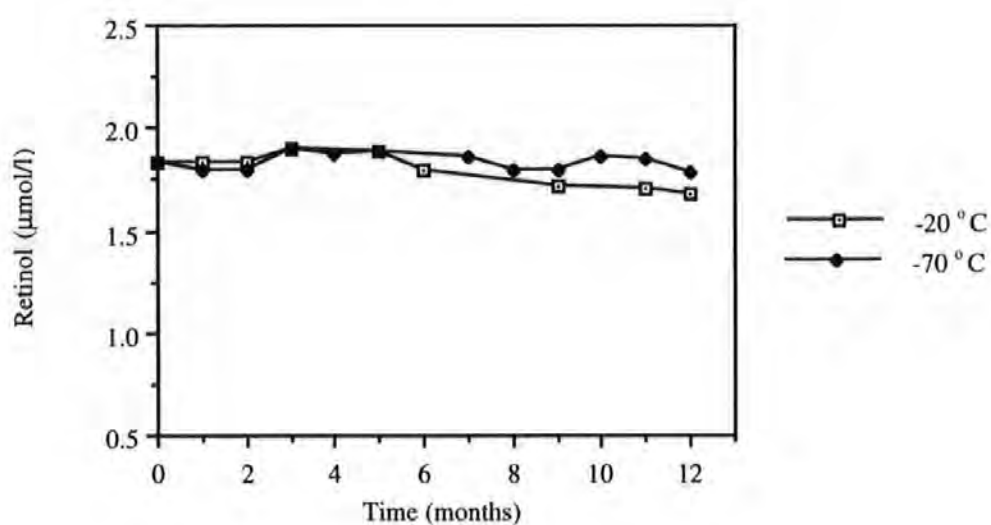
Figure 5.5 *Plasma retinol concentrations in samples stored at -70°C and -20°C for 12 months.*
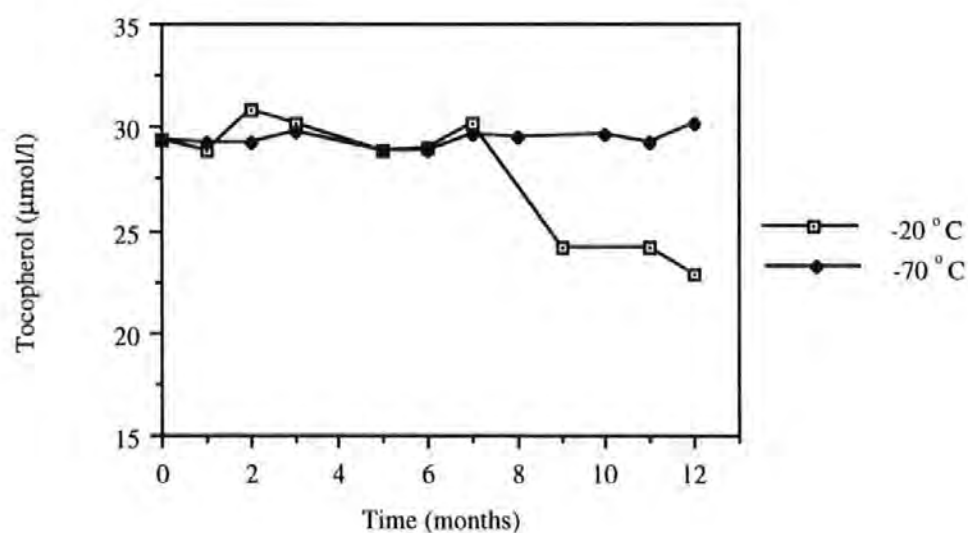
Figure 5.6 *Plasma α-tocopherol concentrations in samples stored at -70°C and -20°C for 12 months.*

5.7 Discussion

Greater than 95% of vitamin A in serum is all-*trans* retinol. Retinol is also transported post prandially in the form of retinyl esters, predominantly as retinyl palmitate with smaller amounts appearing as retinyl stearate, oleate and linoleate (De Leenheer *et al.* 1979). More than 95% of vitamin E activity is represented by α-tocopherol, the remainder occurs in the form of ß and γ-tocopherol (Catignani and Bieri 1983). The method described, quantifies all-*trans* retinol and α-tocopherol, the predominant forms of vitamins A and E in plasma.

During the initial investigations of HPLC methods, three reverse phase methods with different mobile phases were attempted (Thurnham *et al.* 1988; Nierenberg and Nann 1992; MacCrehan and Schönberger 1987). The best results were obtained with the method of MacCrehan and Schönberger (1987). The mobile phase was very simple to prepare and relatively non-hazardous and this was chosen for further work. The mobile phases of the other methods contained more hazardous chemicals, namely tetrahydrofuran and chloroform, resulting in solutions which produced offensive odours and made the handling of these mobile phases unpleasant. Small adjustments in the composition of the mobile phase enabled baseline resolution of all four compounds with an acceptable run time of 11 minutes. Switching wavelengths from 324 nm to 292 nm, after 5.15 minutes, allowed optimum detection of the analytes without any disruption to the chromatographic trace.

The detector response was found to be linear up to at least 12.5 μmol/l for retinol and 220 μmol/l for α-tocopherol and, therefore, encompassed the range expected in plasma samples (1.05-2.8 μmol/l retinol; 12-42 μmol/l α-tocopherol (Teitz 1990)). As proposed by Horowitt *et al.* (1972) and Thurnham *et al.* (1986), the assessment of vitamin E status should take into account the lipid status of the individual. Thus, measurements of serum cholesterol and triglycerides were also made in the patients studied, in order to provide a full assessment of the vitamin status.

The between batch coefficients of variation (retinol 3%, α-tocopherol 5%) were found to be similar to the values reported by MacCrehan and Schönberger (1987) (retinol 4.1%, α-tocopherol 4.7%) and lower than those reported by Thurnham *et al*. (1988) (retinol 10%, α-tocopherol 5.3%) and Nierenberg and Nann (1992) (retinol 9.1%, α-tocopherol 6.7%). This favourable result reflected the high reproducibility of the assay.

The determination of the absolute recoveries of the analytes is made difficult by the fact that the vitamins are bound in lipid-protein associations in plasma. The simple addition of an exogenous substance is, therefore, not a good marker for the recovery of an endogenous substance (MacCrehan and Schönberger 1987). However, the results obtained (94.6% for retinol and 93% tocopherol) are satisfactory, but only provide an indication of the accuracy of the method.

The use of two internal standards compensated for any differences in the analytical recoveries between the vitamins during the extraction stages of the sample preparation. In addition to this, the internal standards also accounted for losses during the sample preparation, variations in injection volume and small changes in detector sensitivity. The assay was 30 times more sensitive for retinol than for α-tocopherol due to the much higher molar absorptivity of retinol. Fluorescence detection would have increased the sensitivity for α-tocopherol, but this was not required as the detection limits were much lower than levels found in plasma associated with vitamin deficiency.

Driskell *et al*. (1985a) reported that there was no trend towards degradation of vitamin A stored for 5 to 8 years at -20°C and that losses during the sample preparation could be prevented by the addition of AA to the extracting solvent (Driskell *et al*. (1985b). Craft *et al*. (1988) also reported no detectable change in retinol and tocopherol concentrations in plasma stored at -20°C for 5 to 15 months and the vitamins were found to be stable for at least 28 months at -70°C. The report of Edmonds and Nierenberg (1993) indicated that retinol and α-tocopherol were stable for at least 5 years at -70°C.

The stability data in this study indicated that there was no deterioration in retinol and α-tocopherol concentrations after 12 months of storage at -70°C, which supported the previous findings. However, a decrease in retinol and α-tocopherol concentrations was observed after 9 months at -20°C in contrast to the previous reports.

The effects of exposure to air and light, on the concentrations of retinol and tocopherol, during the processing of plasma were investigated by Gross *et al.* (1995). No significant differences between three processing procedures were found, a result which indicated that the vitamins would not be adversely affected by 'typical' clinical blood collection and processing procedures. The effects of repeated freezing and thawing of plasma samples have also shown the vitamins to be stable (Zaman *et al.* 1993; Hsing *et al.* 1989). In this study, precautions were taken during the collection of blood samples and during the sample preparation to ensure that samples were protected from the light at all times. In addition, plasma was separated and stored within two hours of the samples being taken, therefore changes in the concentrations of the analytes were unlikely.

In conclusion, the method described enables straight forward, rapid and accurate analysis of retinol and α-tocopherol in plasma, with the advantage that samples can be safely stored for long periods at -70°C prior to analysis.

Chapters 6-7

Patient Studies

6. Free Radical Activity and Antioxidant Vitamin Status During Diabetic Ketoacidosis and Severe Hyperglycaemia

6.1 Introduction

Diabetic ketoacidosis (DKA) occurs when a relative or absolute deficiency of insulin in the presence of counter-regulatory hormones, primarily glucagon, but also catecholamines, cortisol and growth hormone results in hyperglycaemia, ketonaemia and acidosis. The most frequent precipitating factors of DKA are infection, acute illness, omission of insulin injections, missed diagnosis and failure to increase the insulin dosage during times of infection or other forms of insulin resistance (Elamin 1993; Fleckman 1993).

A small number of patients have repeated episodes of DKA where psychological and social problems or unstable family environments are the general underlying causes. These patients are often described as having 'brittle' diabetes and have poor glycaemic control with frequent episodes of DKA (Tattersall *et al.* 1991). The erratic metabolic control leads to poor prognosis and may place patients at increased risk of developing complications (Williams and Pickup 1988).

The autoxidation and glycoxidation reactions of glucose under physiological conditions *in vitro* result in the formation of ketoaldehydes, H_2O_2 and highly reactive oxidants (Hunt *et al.* 1993). Consequently, patients with DKA may be under increased oxidative stress as a result of hyperglycaemia, since plasma glucose concentrations of the order of 42 ± 22 mmol/l, reaching 135 mmol/l in rare instances have been reported (Fulop and Eder 1989).

6.2 Aims

This study was undertaken as a preliminary investigation of the hypothesis that the autoxidation / glycoxidation reactions of glucose may be a sources of ROS *in vivo*. Patients with DKA were chosen for the study, since plasma glucose concentrations approximate to the levels used in the *in vitro* investigations and remain elevated over a number of days. The acute effects of hyperglycaemia on lipid peroxidation and antioxidant vitamins were studied.

6.3 Patients and Methods

The study was given the approval of the Ethical Committee of the South Devon Health Care Trust. Patients with DKA, not necessitating intensive care treatment, were studied. Samples were taken at the time of admission to the Casualty Department at Torbay Hospital and then on a daily basis during the recovery period, after the patients had been transferred to the hospital wards. In order to minimize discomfort and further distress to the patients, samples for the study were taken at the same time as the routine blood samples. The patients were treated with soluble insulin and appropriate fluid replacement therapy.

Blood samples were collected into 10 ml Vacutainer tubes containing EDTA (Becton Dickinson Ltd., Oxford), for the analysis of TBARS, the conjugated diene isomer of linoleic acid and its molar ratio to linoleic acid, together with total plasma fatty acids and plasma vitamins A, E and C (AA+DHAA).

Whole blood was collected into tubes containing separator gel for the analysis of serum cholesterol, triglycerides and uric acid. Blood for the analysis of plasma glucose was collected in tubes containing sodium fluoride. Total serum cholesterol, triglycerides and uric acid were determined using commercially available enzymatic-colorimetric kits, adapted for the Hitachi 747 analyser (Boehringer, Mannheim, Germany). Plasma glucose levels were measured by a glucose oxidase method. The H_2O_2 generated during the analysis was measured directly with electrochemical detection, with a platinum electrode, using a Model GA-1120 Auto and Stat analyser (Clandon Scientific Ltd. UK). The results for plasma bicarbonate, creatinine, urea, arterial blood pH and the presence of ketones were also available from the Chemical Pathology Department at Torbay Hospital.

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6.4 Results

Six patients were studied. The biochemical characteristics of the patients on admission are shown in Table 6.1 and their clinical details are described below:

Patient 1: a 60 year old female, with type I diabetes, with very unstable diabetic control as a result of a psychological upset, was admitted with polyuria and polydipsia. The patient was dehydrated, ketotic and acidotic and unable to eat for 48 hours during the recovery period.

Patient 2: an 86 year old male subject, with a 12 year history of type II diabetes, treated by diet and glibenclamide, was admitted to the casualty department with confusion, polyuria, vomiting, shingles and upper respiratory tract infection. The patient was only mildly dehydrated, ketotic, but not acidotic and was able to eat after 24 hours.

Patient 3: a 38 year old male subject, severely overweight with a BMI of 45, newly diagnosed with type II diabetes and under stress, was admitted to the hospital after 4 weeks of polyuria, polydipsia and weight loss with vomiting. The patient was ketotic and acidotic and able to eat after 24 hours.

Patient 4: an 83 year old female, with newly diagnosed type II diabetes, presented with polyuria, polydipsia and feeling generally unwell due to difficulties in accepting treatment. The patient was ketotic, but not acidotic and was also able to eat on the following day.

Patient 5: a 38 year old female, with type I diabetes of 10 years duration, was admitted with severe oesophagitis and vomiting, which resulted in dehydration and raised plasma glucose concentrations, but not full DKA. The patient was monitored over a 3 day period and was able to eat small amounts of food 24 hours after admission.

Table 6.1 *The biochemical characteristics of the patients on admission to hospital on Day 1.*

	PATIENT					
	1	**2**	**3**	**4**	**5**	**6**
Plasma glucose (3 - 5.5 mmol/l)	28.7	38.1	37.0	37.1	10.8	46.1
Plasma bicarbonate (22 - 32 mmol/l)	18	24.0	15.0	22.0	21.0	28.0
Arterial blood pH (7.35 - 7.45)	7.11	NR	NR	7.38	NR	NR
Creatinine (44 - 124 μmol/l)	113	167	138	148	80	227
Urea (2.5 - 6.6 mmol/l)	10.9	11.9	11.6	12.0	5.6	20.9
Ketones (in whole blood)	++	+	++	+	-	-

The reference ranges for healthy individuals are shown in brackets.
NR signifies no result available.

Patient 6: at the time of the study, a 43 year old female patient with type I diabetes of 14 years duration, with early renal impairment (as indicated by the high values of plasma creatinine and urea, Table 6.1) and a history of extremely severe episodes of hyperglycaemia, without ketosis, was found to have a plasma glucose concentration of 46 mmol/l, a fructosamine concentration of 1284 mmol/l (reference range, 200 - 300 mmol/l) and a glycated haemoglobin concentration >20% (reference range, 4 - 7%) during a routine visit to the diabetic clinic. The patient had no apparent symptoms of dehydration, polyuria or polydipsia and appeared to be feeling in good health. The patient was treated as an outpatient and was monitored daily over a 1 week period, whilst improvements in glycaemic control were advocated.

The initial blood samples were collected between the hours of 4 pm and 5 am, upon admission of the patients to the casualty department (Day 1). The follow-up samples were collected daily, between 9 am and 11 am, during the collection of the routine blood samples in order to minimize any discomfort to the patients. All patients were followed up for three days, one patient was followed up for four days, one for five days and one for six days. The collection of blood samples was more difficult in the older patients, so these individuals were monitored for three days only.

The results are expressed as mean ± SD, unless stated otherwise. Due to the difficulty in obtaining more than six patients for the study, statistical analyses were not performed on the data and the results are presented in descriptive terms. Comparisons were made between Days 1 and 3 in particular, since all patients were followed up for three days.

Glucose and serum lipids

Plasma glucose concentrations were high on admission and remained elevated during the recovery period as shown in Table 6.2 below. Serum triglyceride levels were raised above the reference range in patients 1-4, as shown in Table 6.3. By Day 3, the triglyceride levels had returned to the reference levels in two of these patients, as a result of the intravenous administration of insulin and fluids. Hypertriglyceridaemia was observed in patient 3 on Day 1. Patient 3 was newly diagnosed and had been poorly controlled for several weeks before admission. Serum cholesterol levels were also elevated on admission, but decreased gradually during the recovery period as shown in Table 6.4. The overall changes in triglyceride and cholesterol levels are shown in Figure 6.1 (page 136).

Table 6.2 *Changes in plasma glucose levels.*

			PATIENT				
	1	**2**	**3**	**4**	**5**	**6**	**Mean ± SD**
	Glucose (mmol/l)						
DAY							
1	14.2 *	38.1	15.0	13.6	10.8	46.1	23.0 ± 15.1
2	20.4	3.6	16.6	24.1	6.2	8.3	13.2 ± 8.3
3	10.4	8.1	22.3	25.4	14.0	7.7	14.7 ± 7.5
4	14.6	-	24.0	-	-	7.1	
5	-	-	28.3	-	-	9.7	

Reference range: 3 - 5.5 mmol/l.
* The plasma glucose concentrations on Day 1, in patients 1, 3 and 4, differ between Tables 6.1 and 6.2 as a result of a time delay (of between 0.5-3 hours) between the first samples collected immediately on admission and those collected for this study.

Table 6.3 *Changes in serum triglyceride levels.*

	PATIENT						
	1	**2**	**3**	**4**	**5**	**6**	**Median (range)**
Triglycerides (mmol/l)							
DAY							
1	3.3	2.7	17.3	2.1	1.0	1.8	2.4 (1.6 - 6.8)
2	1.5	2.0	5.6	2.0	1.0	1.6	1.8 (1.4 - 2.9)
3	1.4	1.7	5.7	2.2	1.2	0.4	1.5 (1.0 - 3.7)
4	1.5	-	4.4	-	-	0.5	
5	-	-	4.2	-	-	1.9	

Reference range: 0.8 - 2.0 mmol/l.

Table 6.4 *Changes in serum cholesterol levels.*

	PATIENT						
	1	**2**	**3**	**4**	**5**	**6**	**Mean ± SD**
Cholesterol (mmol/l)							
DAY							
1	10.0	5.2	11.1	6.0	3.6	6.4	7.05 ± 2.9
2	9.0	4.5	7.6	6.6	3.6	4.7	6.0 ± 2.1
3	7.9	3.9	8.1	6.5	3.8	4.9	5.8 ± 1.9
4	7.7	-	7.2	-	-	5.4	
5	-	-	6.9	-	-	5.9	

Reference range: 3.7 - 6.5 mmol/l.

The changes in plasma MDA are shown in Figure 6.2. The levels were markedly raised above the reference range in four subjects on Day 1, with patient 3 exhibiting a seven fold increase above the reference range found in healthy subjects. During the recovery period, the plasma levels of MDA showed a gradual decline in five patients. Interestingly, the changes taking place were similar to the pattern observed for the sum of the serum triglyceride and cholesterol concentrations shown in Figure 6.1. Further investigations revealed that the plasma MDA concentrations were found to correlate positively with serum triglyceride levels (r = 0.93), cholesterol levels (r = 0.55) and with the sum of the triglyceride and cholesterol levels (r = 0.86, Figure 6.3).

Figure 6.4 shows the change in MDA concentrations in relation to the change in serum lipids. In patients 1 and 3, the MDA / triglyceride + cholesterol ratio remained constant despite the large changes in serum lipids that were taking place. In patients 2 and 4, the ratio decreased, whereas in patient 5 the ratio had increased by Day 3, whilst the serum lipid concentrations remained constant.
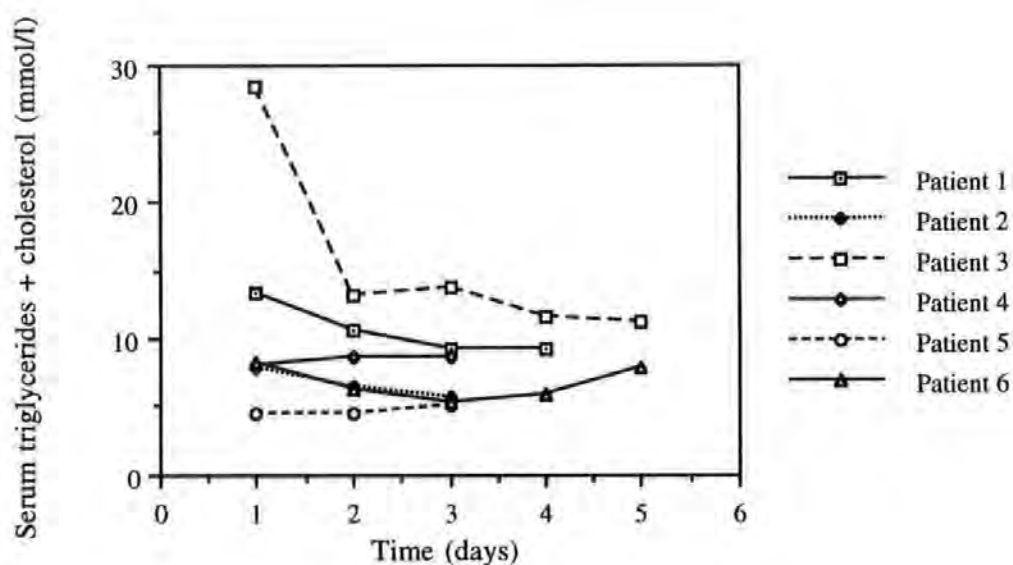
Figure 6.1 *Changes in serum triglyceride + cholesterol levels during the recovery period.*
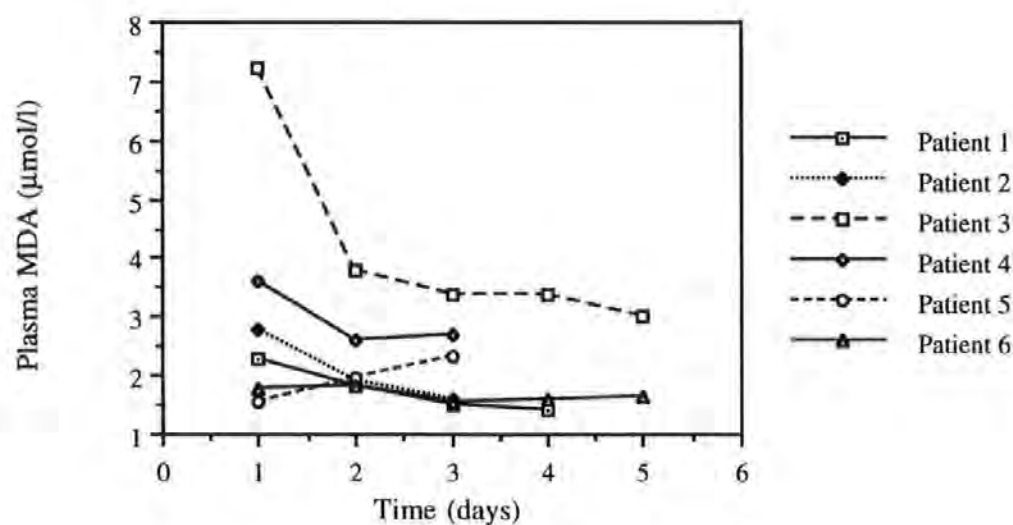
Figure 6.2 *Changes in plasma MDA concentrations during the recovery period. Plasma reference range in healthy subjects: 0.6 - 1.4 µmol/l and in diabetic patients with stable glycaemic control 0.9 - 2.5 µmol/l.*

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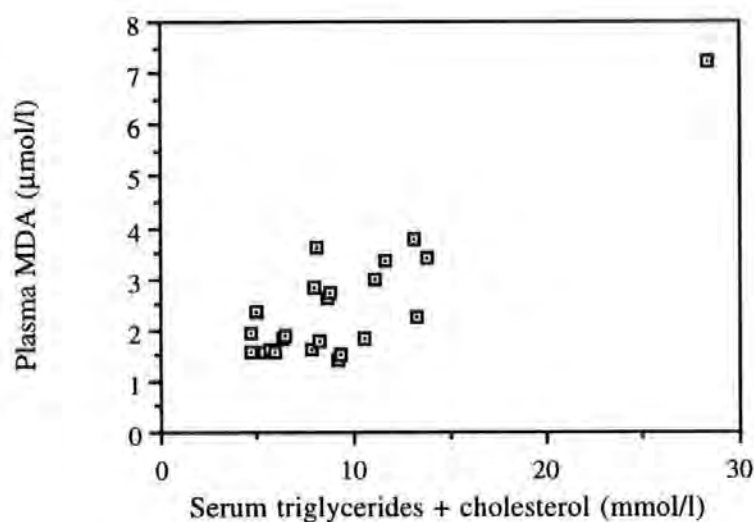
Figure 6.3 *Correlation between plasma MDA concentrations and the sum of serum triglyceride and cholesterol levels (r = 0.86).*
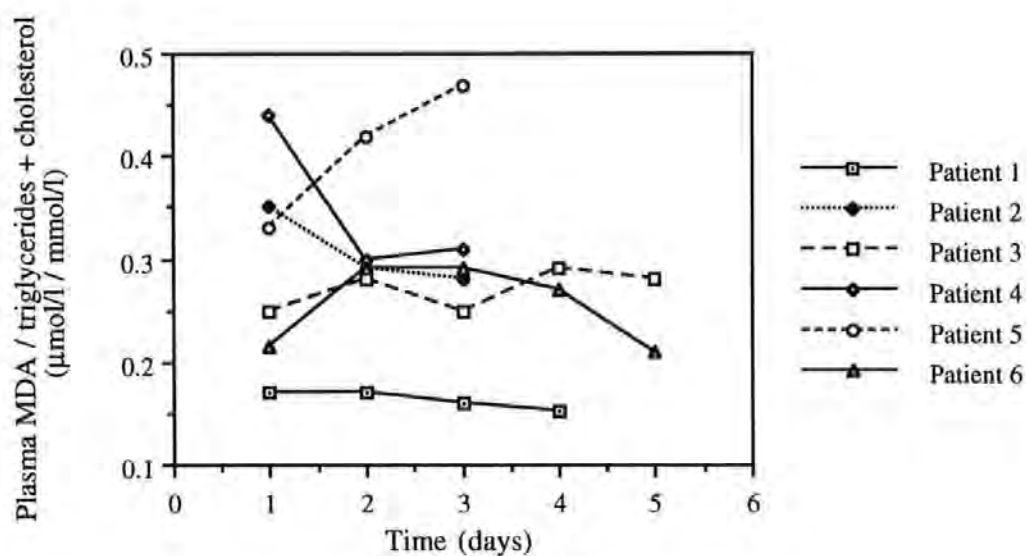
Figure 6.4 *Changes in the MDA / triglyceride + cholesterol ratio during the recovery period.*

Changes in the conjugated diene / linoleic acid ratio are shown in Figure 6.5. The conjugated diene ratio was markedly raised above the reference range in patient 3, but decreased gradually during the follow-up period. Patient 3 was newly diagnosed and the increase in the conjugated diene ratio on Day 1 was possibly due to several weeks of poor metabolic control prior to the study. In the remaining patients, the conjugated diene / linoleic acid ratio remained stable and within the reference range. The changes observed were similar to the changes in the plasma MDA concentrations.

Positive correlations were also found between conjugated diene concentrations and serum triglyceride levels ($r = 0.89$), cholesterol levels ($r = 0.86$) and the sum of the triglycerides and cholesterol levels ($r = 0.96$, Figure 6.6).
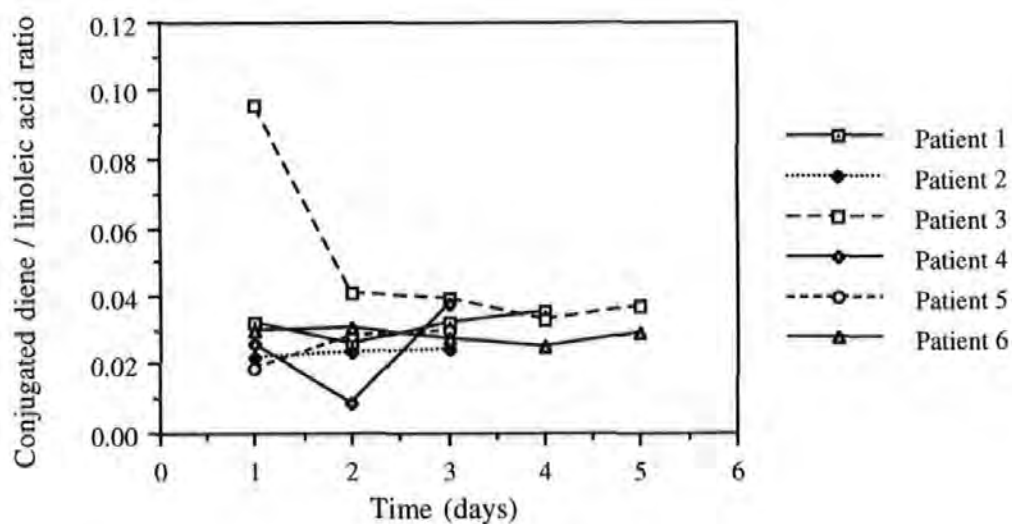
Figure 6.5 *Changes in the conjugated diene / linoleic acid ratio during the recovery period. Reference range 0.009 - 0.032.*
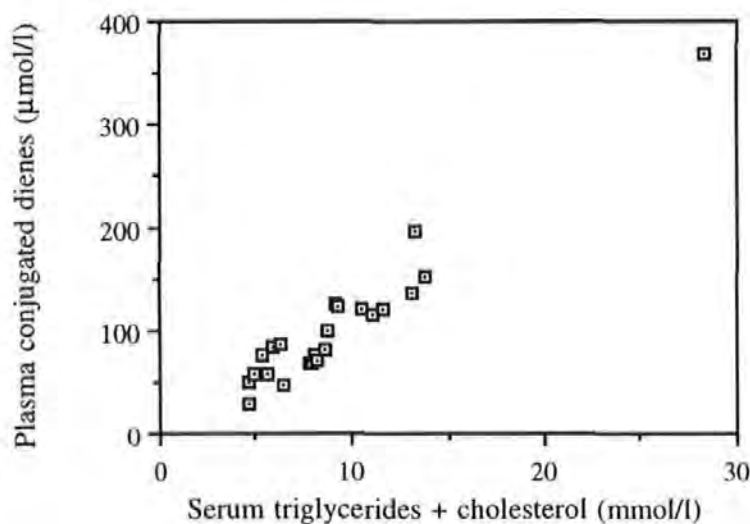
Figure 6.6 *Correlation between plasma conjugated dienes and the sum of the triglyceride and cholesterol levels (r = 0.96).*

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Lipid-soluble antioxidants

The changes in the lipid-soluble antioxidants, retinol and α-tocopherol, are shown in Figures 6.7 and 6.8. Plasma retinol concentrations remained constant in three patients, but decreased in two patients. In patient 6, the plasma concentrations of retinol were raised above the reference range during the follow-up period, a further indication of the mild renal impairment in this patient.

The plasma α-tocopherol concentrations showed a gradual decline during the follow-up period in four patients. In contrast, the α-tocopherol / triglyceride + cholesterol ratios increased during the follow-up period in four patients, but decreased slightly in two patients, as shown in Figure 6.9. The average values on Day 3 (3.4 ± 0.7 μmol/l α-tocopherol / mmol/l lipid (mean $\pm$ SD)) were slightly lower than on Day 1 (3.5 ± 1.1). However, the α-tocopherol / triglyceride + cholesterol ratios remained above the level considered to be the deficiency threshold (1.59 μmol/l α-tocopherol / mmol/l lipid (Thurnham *et al.* 1986), throughout the recovery period, in all of the patients.
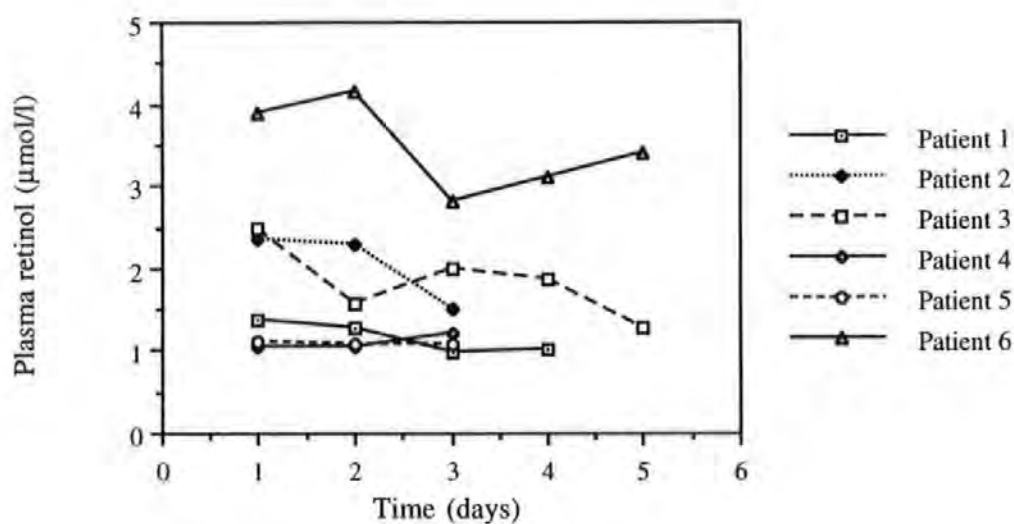
Figure 6.7 *Plasma retinol concentrations during the recovery period. Reference range 1.05 - 2.8 μmol/l (Tietz 1990).*
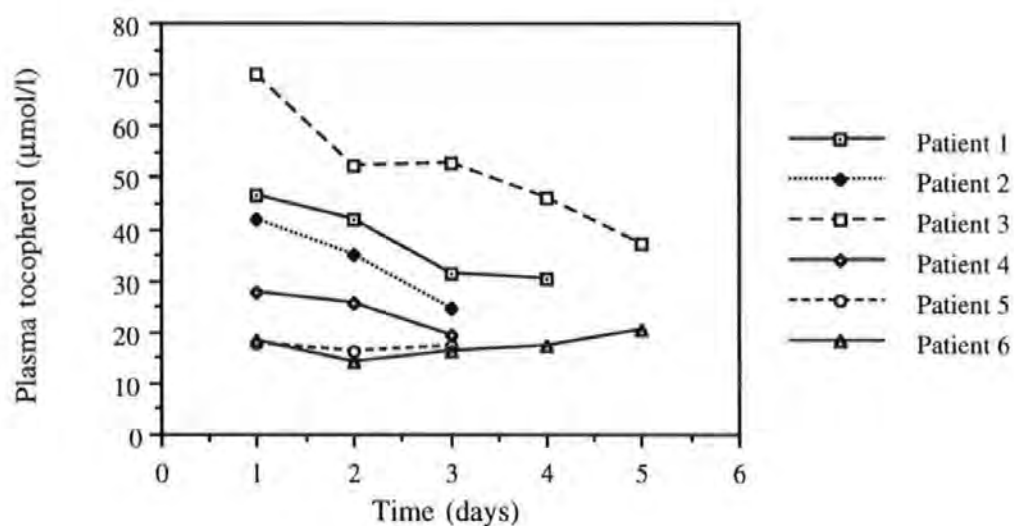
Figure 6.8 *Plasma α-tocopherol concentrations during the recovery period. Reference range 12 - 42 μmol/l (Tietz 1990).*

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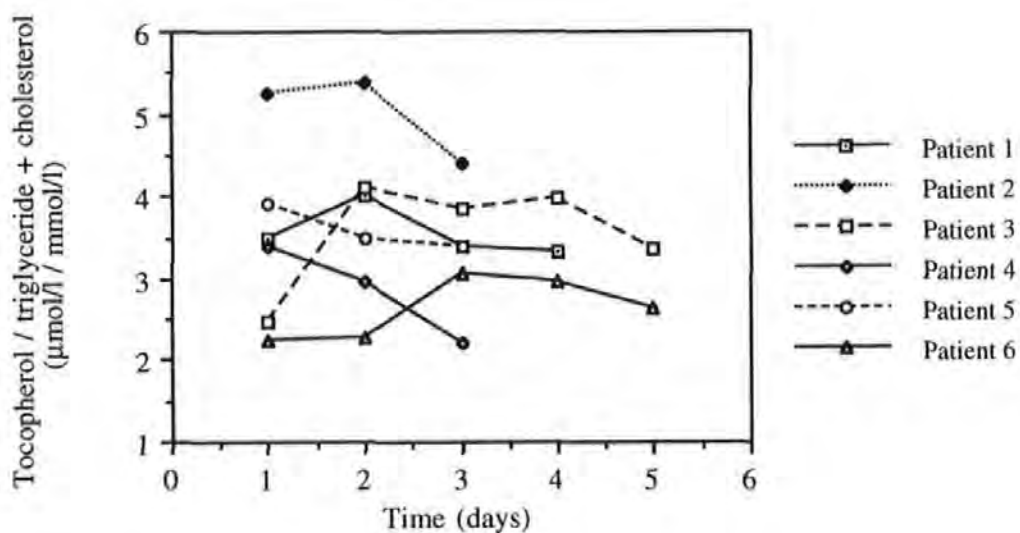
Figure 6.9 *Changes in plasma α-tocopherol / triglyceride + cholesterol ratios (μmol/l α-tocopherol / mmol/l lipid). Values below 1.59 μmol/l α-tocopherol / mmol/l lipid were considered to be indicative of vitamin E deficiency (Thurnham et al. 1986).*

Table 6.5 *Plasma DHAA and DHAA/AA ratio in five patients during the recovery period.*

	PATIENT						
	1	2	3	4	6*	Mean ± SD	
DHAA (DHAA/AA) (μmol/l)							
DAY							
1	0.6 (0.009)	2.2 (0.03)	13.5 (0.19)	0 (0)	0 (0)	3.3 ± 5.8	(0.05 ± 0.08)
2	3.1 (0.07)	0.6 (0.008)	0.3 (0.01)	9.2 (0.25)	0 (0)	2.6 ± 3.9	(0.07 ± 0.1)
3	1.8 (0.04)	2.4 (0.04)	1.5 (0.05)	1.9 (0.06)	2.6 (0.08)	2.0 ± 0.5	(0.05 ± 0.02)
4	0.3 (0.01)	- -	0 (0)	- -	1.1 (0.04)		
5	- -	- -	6.5 (0.34)	- -	2.0 (0.07)		

The concentration of DHAA in healthy individuals varies from 2.7 ± 2.5 to 31.8 ± 4.8 μmol/l (mean ± SD) (see Table 1.6 page 62).

* Sample not available for patient 5.

Water-soluble antioxidants

Changes in the concentrations of the main water-soluble antioxidants in plasma, uric acid and AA are shown in Figures 6.10 and 6.11. The concentrations of uric acid were increased above the reference range in three patients on Day 1. During the recovery period, a gradual decline in the levels of uric acid were observed in five patients, with values approaching the reference range by Day 3 in three patients. On average, the plasma levels of uric acid had been reduced by 37% ± 15% by Day 3. In patient 6, the levels of uric acid remained at the upper end of the reference range during the period of observation, whilst in patient 5 the levels were below the reference range.

Similar changes in the concentrations of AA were found. The initial concentrations of AA were high, but showed a gradual decline during the recovery period, approaching the lower limits of the reference range in two patients by Day 2. By Day 3, plasma ascorbate levels had been reduced by an average of 38% ± 15%. Table 6.5 shows the concentration of DHAA and its ratio to AA in five patients. Large intra- and inter-patient variability in the levels of DHAA and the DHAA/AA ratio were observed. The concentration of DHAA ranged from 0 - 13.5 μmol/l, with an overall mean ± SD of 2.5 ± 3.5 μmol/l; the DHAA/AA ratio ranged from 0 - 0.34 (0.06 ± 0.09). These results were similar to the ranges found in diabetic subjects with stable glycaemic control (DHAA: 0 - 14.7 μmol/l (2.8 ± 3.1 μmol/l); DHAA/AA ratio: 0 - 0.42 (0.09 ± 0.09, $n = 29$)) and lower than those found in a small number of non-diabetic subjects (DHAA: 1.8 - 27.2 μmol/l (12.8 ± 8.7 μmol/l); DHAA/AA ratio 0.06 - 0.42 (0.18 ± 0.1, $n = 6$)) who were monitored during the method validation.

An estimate of plasma TRAP was made by calculation (although plasma thiols were not measured) according to the formula (2.0 [vitamin E] +1.3 [uric acid] + 1.7 [ascorbic acid]) (Ceriello *et al.* 1997). The mean ± SD values obtained on Days 1 - 3 were: 829 ± 350, 651 ± 227, 538 ± 167 μmol/l respectively. The range in healthy subjects was reported to be 669 ± 12 μmol/l (Ceriello *et al.* 1997). Thus, the calculated TRAP values were elevated on Day 1 and showed an overall decrease of 34 ± 16%, by Day 3, in the patients studied.
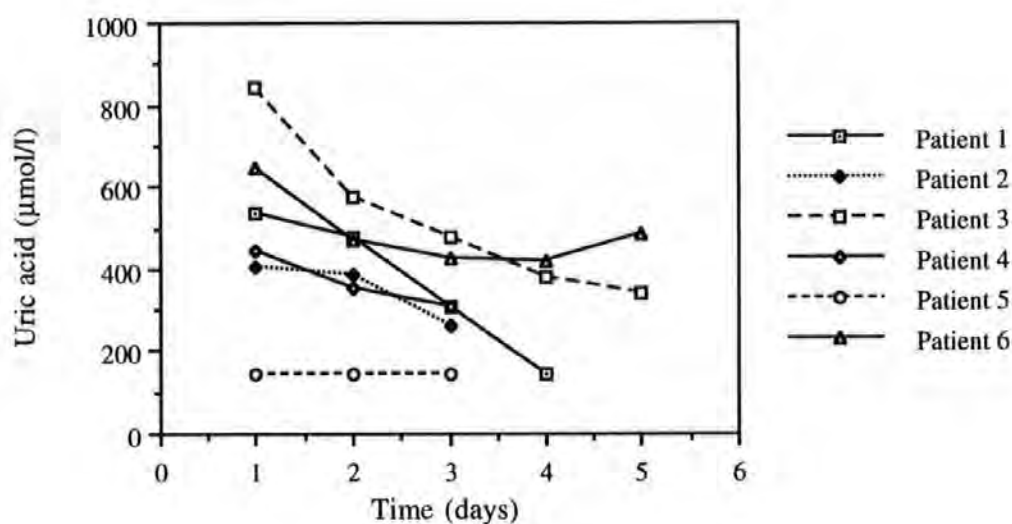
Figure 6.10 *Uric acid concentrations during the recovery period (reference range 180-420 μmol/l).*
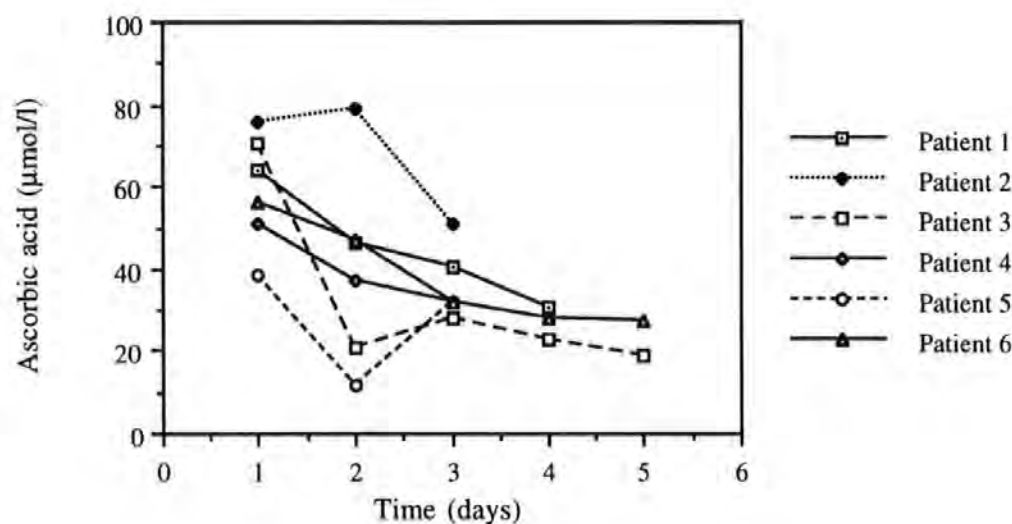
Figure 6.11 *Plasma AA concentrations during the recovery period (reference range 28-85 μmol/l (Tietz 1990)).*

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6.5 Discussion

The purpose of this study was to investigate the acute effects of markedly raised plasma glucose concentrations on free radical activity *in vivo*. This was based upon the hypothesis, from numerous *in vitro* studies, that the autoxidation and glycoxidation reactions of glucose are sources of ROS. Patients with very poor glycaemic control were monitored over a number of days, during the normalization of plasma glucose levels, in order to gain an insight into the effect of hyperglycaemia on lipid peroxidation. Patients with DKA were chosen as subjects for the study, since the plasma glucose concentrations in these patients approximate to the levels used in the *in vitro* investigations (i.e., 25 mmol/l (Hunt and Wolff 1991; Hunt *et al.* 1993)) and remain elevated for a several days. However, due to the large metabolic changes that occurred in the patients during the episodes of DKA, it was not possible to determine clearly the effects of plasma glucose. Nevertheless, some interesting insights into free radical activity during DKA and severe hyperglycaemia have been gained from this study.

Diabetic ketoacidosis represents a very large metabolic disturbance. In the absence of insulin, the rates of glycogenolysis and gluconeogenesis are enhanced, raising the serum glucose concentrations to levels which cause osmotically driven polyuria and polydipsia. The consequent loss of water and electrolytes from the kidneys causes dehydration, which is often further exacerbated by vomiting. Due to the lack of insulin, excessive lipolysis results in high levels of serum FFAs which are metabolized to the ketoacids, ß-hydroxybutyrate and acetoacetate. The accumulation of these acids and their dissociation, with the release of hydrogen ions, lowers the serum bicarbonate concentration causing metabolic acidosis, which if left uncorrected leads to coma and death.

During the treatment of DKA, insulin therapy causes a fall in plasma FFAs and ketone bodies by inhibiting lipolysis and ketogenesis. The therapy is aimed at reversing the underlying ketoacidotic state and not, initially, at normalizing the plasma glucose concentrations. Consequently, the plasma glucose levels fluctuated, but remained elevated during the follow-up period, whilst the serum triglyceride and cholesterol levels, which were

markedly raised above the reference range on Day 1, declined steadily during the follow-up period as a result of the treatment. Interestingly, hypertriglyceridaemia was observed in patient 3 on Day 1. Patient 3 was poorly controlled for several weeks before admission. Raised serum triglycerides in this patient were secondary to the uncontrolled diabetes, a finding which is not uncommon in patients with DKA (Fulop and Eder 1989).

As reported in the literature, the main precipitating factors leading to the development of DKA, namely infection and treatment errors (Fleckmann 1993) were found in the patients studied. Patient 1, classed as having 'brittle' diabetic control, was admitted during an unpredictable episode of DKA, the cause was attributed to psychological upset as a result of an unstable family environment. In patient 2, an infection was the underlying cause of the ketotic episode. Patients 3 and 4 were newly diagnosed, one severely overweight and under stress, the other experienced difficulties in accepting treatment which resulted in DKA. Patients 5 and 6 were included in the study, because of severe hyperglycaemia, without true DKA. Indeed, patient 6 was a unique subject with a history of extremely severe episodes of hyperglycaemia without ketosis.

Lipid peroxidation

The concentrations of MDA measured on Day 1, in three patients, were two to four fold greater than the range found in healthy individuals and in diabetic patients with stable glycaemic control. In one patient, the MDA levels were increased seven fold above the range found in healthy individuals. These results were much higher than any results that had been previously encountered during the method validation. All of the samples collected from each patient were analysed on the same day under identical conditions in order to minimize the variation due to the method itself. This factor was not therefore responsible for the variation that was observed in the results.

Initially, it was suspected that interference in the assay may have caused the high results, possibly due to the presence of ketone bodies in this group of patients. The addition of acetone, ß-hydroxybutyrate or acetoacetate to plasma or blank samples, produced a minimal increase in the results, within the variation of the method, indicating that the ketone bodies were not causing any interference in the assay nor contributing to the abnormally high results. Excessive lipid peroxidation during the acid-heating stage of the TBAR test has been reported in samples from patients with hyperlipidaemia (Chirico *et al.* 1993). This was also a possibility in this group of patients due to the high lipid content of the samples, which may have amplified lipid peroxidation during the assay, producing artefactually elevated results. However, the conjugated dienes were also elevated, especially in patient 3, and showed similar changes to the MDA levels, supporting the reliability of the TBAR results. Interestingly, as serum concentrations of triglycerides and cholesterol were normalized, MDA and conjugated diene levels also declined towards the reference ranges, suggesting that the serum lipid peroxide levels were proportional to the serum lipid levels. Positive correlations were found between plasma MDA and serum triglyceride ($r = 0.93$) and cholesterol levels ($r = 0.55$) and also between conjugated dienes and serum triglycerides ($r = 0.89$) and cholesterol ($r = 0.86$). Thus, it appears that the increase in plasma MDA and conjugated diene concentrations was proportional to, and may be explained by the increase in serum lipids.

One other study has investigated lipid peroxidation in patients with DKA (Faure *et al.* 1993). Samples were collected on two occasions, once during admission to hospital before treatment with insulin and again after 14 ± 3 days when stable glycaemic control had been reached. The results indicated that TBARS were significantly increased during DKA, but after glycaemic improvement the concentrations had approached the reference values. The results of this study support this finding.

During the development of DKA, reduced insulin levels are accompanied by excessive lipolysis and release of FFA by adipose tissue. Increases in plasma FFA may, therefore, be partly responsible for the increases in MDA and conjugated dienes that were observed, since FFA attached to albumin (although a strong antioxidant) may be more susceptible to free radical attack than fatty acids within lipoproteins. Reaven (1988) showed that the progression from non-diabetic through IGT to Type II diabetes was accompanied by a progressive increase in circulating FFA. Interestingly, Niskanen *et al*. (1995) have shown that TBARS also increase from non-diabetes to IGT and diabetes. A recent study has shown that an infusion of a triglyceride emulsion (Intralipid), in healthy individuals, resulted in an increase in plasma FFA levels, which in turn were accompanied by an increase in plasma TBARS (Paolisso *et al.* 1996). Thus, FFA concentrations may have implications on the plasma levels of TBARS in diabetic patients, factors which require further investigation.

Regardless of the pathways by which MDA levels were elevated in these patients, or the proportion of MDA to serum lipids, one factor for concern was that plasma MDA concentrations were markedly raised above the reference range and in one case seven-fold above the average concentration found in healthy subjects. During the breakdown of lipid peroxides, a range of end products are produced, which include saturated and unsaturated aldehydes and ketone products, some of which are cytotoxic and can cause tissue damage (Esterbauer 1993). On Day 1, plasma MDA and conjugated dienes were at their highest levels, whilst α-tocopherol / lipid ratios were at their lowest levels in those patients who experienced the greatest changes in serum lipids. The accumulation of lipid peroxides and their subsequent decomposition has the potential for inducing protein damage in these

patients. Furthermore, frequent episodes of ketosis and elevated levels of acetoacetate have been put forward as risk factors for the oxidative modification of LDL (Jain *et al.* 1998). Thus, patients with severe hyperglycaemia or DKA were most likely to be at increased risk from cellular damage due to oxidative stress, during the large increase in serum lipids and the associated increase in lipid peroxides, and also possibly due to the presence of elevated acetoacetate levels, prior to their admission to hospital.

Lipid soluble antioxidants

Plasma retinol concentrations appeared to be stable during the recovery period. Plasma α-tocopherol concentrations were raised on Day 1, but decreased by Day 3. The α-tocopherol / lipid ratios were lowered slightly in patients who had the largest increase in serum lipids on Day 1, thereafter the ratios increased during the recovery period. However, the α-tocopherol / lipid ratios remained above 1.59 μmol/l / mmol/l lipid, the value considered to indicate vitamin E deficiency (Thurnham *et al.* 1986), suggesting that the patients should not be at increased risk from oxidative stress during the recovery period.

Water-soluble antioxidants

The concentrations of the water-soluble antioxidants, uric acid and AA, were found to be initially high on admission and showed significant decreases during the recovery period. Dehydration, on admission, could have been a contributing factor to the elevated levels and, therefore, the fluid replacement therapy could have been responsible for the lowering of the water-soluble antioxidants by haemodilution or by improving renal perfusion and increasing urinary losses. Also, intracellular volume repletion may have enhanced a redistribution of AA into cells, contributing to the gradual decline. Unfortunately, the haematocrit was omitted from the sample preparation to account for haemato-concentration related analytical errors. However, during the course of the treatment, approximately 6 litres of fluids were administered to the patients according to their individual requirements, over a 24-48 hour period. After this period of time, patients would normally be rehydrated, but the decline in uric acid and, in particular, AA continued after 48 hours suggesting that other factors may have been involved.

Ascorbic acid is a major antioxidant in plasma and the first to be consumed during an oxidative insult (Frei *et al.* 1988). The continued decline in AA observed after 48 hours in three patients, may have been as a result of its antioxidant activity. In its antioxidant role, AA is oxidized to DHAA. An increase in the ratio DHAA/AA may, therefore, be indicative of oxidative stress (Jennings *et al.* 1987b). Thus, DHAA was measured in order to provide further insight into the antioxidant function of AA.

The DHAA concentration and DHAA/AA ratio were found to increase and decrease, in all of the patients, at different times during the follow up period (Table 6.5). A large variation in the levels of DHAA were found within and between the patients, but no trends were observed. However, when the results from this study were compared with the values obtained from diabetic subjects with stable glycaemic control and with non-diabetic subjects, who were monitored during the method validation, no differences in the DHAA concentration or the DHAA/AA ratio were found. Consequently, the results from this study suggested that oxidative stress (assessed by the DHAA/AA ratio) was not increased in

patients with DKA. Attempts to compare the results from this study with published studies, in order to gain a further insight into the changes in the ratio under different conditions, are hindered by the inherent problems associated with the determination of DHAA. These are largely due to the instability of DHAA, which has a reported half-life of between 15 - 100 minutes (Baker *et al.* 1983; Bode *et al.* 1990) and also since DHAA is measured indirectly. Consequently, there is wide variation in the reported DHAA values in plasma (Table 1.6, page 62). Thus, further evaluation of the DHAA/AA ratio, as an indicator of oxidative stress is necessary. Measuring the DHAA/AA ratio during a known free radical insult, e.g., during smoking, may have provided a clearer indication of the changes in the ratio during known oxidative stress and might have proved helpful in interpreting the results from this study. Furthermore, simultaneously measuring the ratio of allantoin, the oxidation product of uric acid and the allantoin / uric acid ratio — although technically more demanding — may have provided an additional marker of oxidative stress, since allantoin and uric acid are more stable than DHAA and AA. However, oxidative stress did not appear to increase during the recovery period, although, it was difficult to determine from these results whether the decline in AA was due to an increase in its consumption as an antioxidant.

The plasma concentrations of AA also reflect dietary intake. Five patients were able to consume food after 24 hours during the recovery period, although intakes were not assessed in this study. However, the decline in AA was too rapid to be as a result of a poor nutritional supply. Schorah *et al.* (1996) measured the plasma concentrations of AA and DHAA in acutely ill patients requiring intensive care treatment for various reasons, including accidental injury, surgery and sepsis. The levels of AA were found to be considerably lower in the acutely ill patients compared with healthy controls; the lowest concentrations were found in those patients most severely ill. It was suggested that the low concentrations of AA occurred as a result of the acute-phase response, involving increased synthesis and release of the antioxidant proteins in plasma, which was due to the severity of the illness and not as a result of age, dietary intake or treatment differences (Schorah *et al.* 1996). Thurnham (1997) has also recently discussed the association between the rapid reduction in plasma ascorbate and the acute-phase response.

In summary, multiple factors were probably responsible for the decline in AA observed in this study. It is possible that the initial decline, during Days 1 and 2, was due to the effect of fluid and insulin therapy, leading to rehydration and possibly increasing cellular uptake of AA. The continued decline in AA observed after 48 hours, may have been as a result of its antioxidant properties, although it was difficult to determine this from the ratio of DHAA/AA. Additionally, an acute-phase response as a result of the metabolic trauma of the DKA episode may have contributed to the decrease in AA. However, regardless of the mechanisms responsible for the reduced levels of AA, the AA levels reached the lower limits of the reference range in two patients and were close to those of deficiency (<11 μmol/l; Tietz 1990) in one of the patients studied. Frei *et al.* (1989) have shown that once all of the AA in plasma has been consumed, lipid peroxidation increases thereafter. Since on average, the plasma urate levels decreased by 37% and the ascorbate levels decreased by 38% by Day 3 (and also the calculated TRAP values), the potential for patients to be under increased oxidative stress during DKA was present. However, despite the lowering of the water-soluble antioxidant capacity, plasma α-tocopherol / lipid ratios increased during the recovery period in the patients with the largest changes in serum lipids, providing adequate lipid-soluble antioxidant protection, since plasma MDA and conjugated diene levels were at their lowest. Furthermore, during the recovery period, plasma glucose levels were still elevated. Thus, any increase in oxidative stress due to the presence of glucose was effectively counter-balanced by the consumption of urate and ascorbate. Other plasma components, such as the antioxidant proteins, may have contributed to the preservation of α-tocopherol and protection of plasma lipids. Measurement of plasma TRAP values might have provided a useful indication of the overall changes in the antioxidant capacity in this study. Nevertheless, the results indicated that the plasma antioxidant defences were very robust, during the recovery period, in spite of the severe metabolic disturbances that were taking place.

Glucose and lipid peroxidation

The large metabolic changes that were taking place made it difficult to determine the effects of hyperglycaemia on lipid peroxidation in these patients. Further studies, using more specific markers of free radical activity, are necessary in order to give a clearer indication of presence of the autoxidation and glycoxidation reactions and their effects on levels of oxidative stress and lipid peroxidation *in vivo*.

The autoxidation and glycoxidation reactions of glucose are separate pathways by which glucose may lead to the formation of free radicals *in vivo*. However, it may be difficult to distinguish which pathway contributes more to the generation of ROS during poor glycaemic control. The *in vitro* studies indicate that the relative rates of oxidation of glucose and Amadori adducts vary according to the glucose and phosphate buffer concentrations that are used. At high concentrations of glucose and phosphate buffer, glucose was found to be the primary source of oxidants and AGEs such as CML, whilst at buffer concentrations similar to those *in vivo*, Amadori adducts were the main sources of CML (Wells-Knecht *et al.* 1995). Thus, at normal glucose concentrations *in vivo*, Amadori adducts are probably the main sources of ROS and AGEs, whereas at higher glucose concentrations both Amadori adducts and glucose are potential sources of glycoxidation products. However, the relative contribution of Amadori adduct and glucose concentrations to oxidative stress *in vivo* may be dependent upon other factors such as metal ion availability or other free radical reactions. The reactions may also be site-specific and localized in regions with higher levels of oxidative stress (Wells-Knecht *et al*. 1995; Baynes 1996). Indeed, the multifactorial nature of oxidative stress was illustrated by patient 6 in this study. Interestingly, patient 6 was monitored during an earlier study when levels of glycaemic control were stable. During that period of observation, the plasma conjugated diene / linoleic acid ratio was two-fold greater than the ratio found in this study. Thus, factors other than glucose concentrations may modulate the contribution of glycoxidation and autoxidation reactions to oxidative stress *in vivo*. Numerous other sugars are also precursors of AGEs such as CML. Hence, the measurement of CML may be valuable as a general indicator of oxidative damage in studies with diabetic patients, since CML is a major product of oxidative modification of glycated

proteins, generated by several pathways (Fu *et al.* 1996; Schleicher *et al.* 1997).

A recent study, in patients with type II diabetes, has shown that after three days of constant intravenous glucose and insulin infusion, to maintain glycaemia within the normal range, erythrocyte MDA concentrations fell significantly (Peuchant *et al.* 1997). The decrease in MDA levels was attributed to a reduction in the formation of ROS by autoxidation reactions as a result of the normalization of plasma glucose concentrations. However, hyperglycaemia may induce oxidative stress in erythrocytes in a number of ways. These include: increased glycation of SOD, resulting in decreased enzyme activity and increased formation of $O_2^{\cdot-}$; increased formation of ROS may cause the release of iron from haemoglobin, causing a further increase in oxidative stress (Virgili *et al.* 1996); the production of 15-hydroxy-eicosatetraenoic acid (15-HETE) by lipoxygenases is also augmented by hyperglycaemia and may be a further source of ROS, since inhibition of lipoxygenases decreases lipid peroxidation by 30% in erythrocytes *in vitro* (Rajeswari *et al.* 1991). Clearly, more sensitive methods for the measurement of free radicals are necessary in order to confirm the presence of the autoxidation and glyoxidation reactions *in vivo* and to establish their contribution to the development of oxidative stress in diabetes. Peuchant *et al.* 1997, did however show that normalizing plasma glucose concentrations was beneficial for reducing erythrocyte MDA levels.

The autoxidation and glycoxidation reactions of glucose are dependent upon the presence of transition metal ions. Metal ions are also powerful catalysts in other free radical reactions such as those of lipid peroxidation. In patients with diabetes, body iron stores appear to be associated with blood glucose and insulin concentrations (Tuomainen *et al.* 1997). Furthermore, serum ferritin levels are elevated in diabetic patients with poor glycaemic control; disordered iron metabolism may, therefore, be common in diabetes (Cutler 1989; Gallou *et al.* 1994b). Thus, iron status may be a crucial factor linking autoxidation, glycoxidation, and lipid peroxidation reactions in diabetes and future studies may need to take this into account.

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6.6   Conclusion

This study has shown that in patients with DKA and severe hyperglycaemia, large increases in serum lipids were accompanied by an increase in plasma lipid peroxides. In particular, lipid peroxide levels were markedly elevated and α-tocopherol / lipid ratios decreased in those patients with the largest changes in serum lipids. Consequently, the increase in peroxidizable substrate and high levels of lipid peroxides may be factors for concern in these patients. Patients appear to be at risk from oxidative stress during the metabolic disturbances which lead to severe hyperglycaemia and DKA, due to the increase in peroxidizable substrate, since the accumulation of products of lipid peroxidation are potentially detrimental to human health.

During the recovery period, the water-soluble antioxidants were declining rapidly in the presence of elevated and potentially pro-oxidant glucose concentrations, whilst interestingly, the α-tocopherol / serum lipid ratios increased in the majority of patients and markers of lipid peroxidation were stable. Hence, the results indicated the resilient nature of the plasma antioxidant defences, during the recovery period, in spite of the severe metabolic disturbances that were taking place.

7. The Effects of a Very Low Calorie Diet and Intensive Conventional Dietetic Therapy on Cardiovascular Risk Factors and Indices of Oxidative Stress in Obese Patients

7.1 Introduction

Very low calorie diets are used in the treatment of obesity to produce rapid weight loss whilst preserving vital lean body mass (reviewed by NTFPTO 1993). Many clinics in general practices throughout the UK have used the VLCD 'Lipotrim' for the treatment of obesity in non-diabetic and diabetic patients. Patients commencing on the Lipotrim VLCD programme are required to attend weekly as part of the programme, in order to monitor progress and for compliance with the diet. During the weekly sessions, patients are able to meet as a group for support, encouragement and education in the principles of long term weight management after weight loss. A clinic was started at Torbay Hospital to in order to monitor closely diabetic patients starting the Lipotrim VLCD programme.

The treatment of newly diagnosed diabetic patients involves dietary advice. Patients normally complete a 5 day food record which is sent to the dietician in advance of their visit. At the time of their appointment, patients spend 10 to 15 minutes with a dietician. The advice offered is tailored to suit the food and nutrient requirement of the individual, with emphasis on weight reduction in obese subjects. Three to 4 follow up visits would be arranged at 6-8 weekly intervals, the patients would then be referred back to their physicians for further treatment. The weekly group support was an important feature of the VLCD programme and indicated the need for diabetic patients receiving conventional dietetic advice to have the same support available. Thus, a clinic offering conventional, but intensive, dietetic advice was set up to run parallel with the Lipotrim clinic, enabling a comparison between the two dietary interventions to be made.

Weight loss in obese type II diabetic subjects produces improvements in cardiovascular risk factors and glycaemic control (Henry et al. 1985; Uusitupa et al. 1990b; Wing et al. 1991). However, VLCDs or low fat diets may affect fat-soluble vitamin intake with the potential of

compromising antioxidant protection, especially in diabetic patients — a group in which vitamin status is already altered as a result of the diabetes. Since the prevalence of obesity is increasing, the use of VLCDs may increase in the future. As there have been no reports of the effects of such diets on oxidative stress in type II diabetic subjects, this provided the impetus for investigating these factors in this study.

7.2   Aims

The aims of this study were to assess the safety and efficacy of a VLCD and intensive conventional dietetic therapy (ICD), in obese type II diabetic and obese non-diabetic patients, on cardiovascular risk factors and indices of oxidative stress, over both short term (0 - 6 months, during the acute weight loss phase) and long term (12 months, during weight maintenance).

7.3   Patients

The study was approved by the Ethical Committee of the South Devon Healthcare Trust and all subjects gave written voluntary consent after the nature of the study had been explained. Obese type II diabetic patients were recruited from district diabetic clinics and obese non-diabetic subjects, for the control groups, were recruited by advertisement from the community and from general practices by referral from their physicians. The recruitment period lasted 10 months during which time 84 individuals attended one of a series of discussion groups explaining the two treatment options. The subjects were then allowed to select either the VLCD or the ICD therapy in order to maximize compliance.

Initially, 23 patients were recruited into the VLCD non-diabetic control group, 23 into the VLCD diabetic group, 21 into the ICD diabetic group and 17 into the ICD non-diabetic control group. Of these patients, 20 subjects failed to attend after the initial discussions, 6 patients pursued the VLCD for less than 1-2 weeks and 6 patients attended the ICD group

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for less than 4 weeks. Consequently, 32 individuals were not committed to the study, however, their weights were monitored at the end of the study period as a further control group.

The remaining 52 patients who were recruited into the study agreed to attend weekly during the acute weight loss phase (0-6 months) and then every 1-2 months during the weight maintenance phase, for the duration of the study (3 years). Three years was chosen as an acceptable time for patients on both dietary regimes to achieve their target weights. During the first months of the study, two patients in the VLCD non-diabetic group and one in the VLCD diabetic group initially attended the respective ICD groups. These patients' data was included in the respective VLCD groups only and baseline was taken as the start of the VLCD. Due to the non-compliant subjects, the number of patients recruited into the ICD control group was reduced to 5 and their data has been omitted from the main statistical analysis. A summary of the final patient groups and their associated medical conditions is shown in Table 7.1 and the protocol for the study is outlined in Figure 7.1 (page 164).

All patients were given a preliminary medical examination in order to screen for any condition which might contraindicate dieting for a prolonged period of time. Included in this examination were height, weight, waist/hip, blood pressure measurements, chest and heart examinations. Blood samples were also taken for the analysis of serum lipids, glucose and for hepatic and renal function tests before dieting. For inclusion in the study, subjects were between 25 and 70 years of age, had a BMI of 30 or above, or a waist hip ratio > 0.8 (females), > 1.0 (males). Patients with hypertension, hyperlipidaemia or gout were included in the study. Patients with any severe intercurrent illness such as myocardial infarction, cerebro-vascular events or a history of eating disorders such as anorexia nervosa were excluded from the study.

Table 7.1 *Characteristics of the patient groups at baseline.*

Group	Number	M/F	Age	Medication		Medical Conditions	
VLCD Non-Diabetic Subjects	18	6/12	50 ± 9	Analgesics	2	Angina	2
				Beta-blocker	2	Arthritis	2
				Calcium antagonist	2	Hypertension	3
				Diuretic	4	Hypothyroidism	2
				Thyroxine	2	Multiple sclerosis	1
VLCD Diabetic Subjects	15	7/8	53 ± 6	Beta-blocker	2	Hyperlipidaemia	7
				Bezafibrate	1	Hypertension	8
				Calcium antagonist	2	Myocardial infarction	1
				Diuretic	3		
				Insulin	6		
				Metformin	2		
				Sulphonylurea	5		
ICD Diabetic Subjects	14	3/11	57 ± 8	ACE inhibitor	1	Hyperlipidaemia	6
				Beta-blocker	2	Hypertension	7
				Bezafibrate	1	Hyperthyroidism	1
				Insulin	6	Nephropathy	1
				Metformin	1		
				Sulphonylurea	3		
				Thyroxine	1		
ICD Non-diabetic Subjects	5	3/2	59 ± 11	Beta-blocker	3	Hyperlipidaemia	1
				Diuretic	1	Hypertension	3

7.3.1　Very low calorie diet group

The VLCD, Lipotrim, was used in this study. This was supplied by the Howard Foundation Research Ltd. (Downing Park, Station Road, Swaffham, Bulbeck, Cambridgeshire, UK) and a weekly supply was purchased by each patient during their visit to the hospital. Lipotrim was formulated in accordance with the recommendations of the Department of Health and Social Security's Committee on the Medical Aspects of Food Policy Report (DHSS 1987). The composition of the diet is listed in Appendix 4.

Lipotrim was a nutritionally complete formula and was used as a total food replacement in the form of a liquid drink or a flapjack. For women, the diet provided a total daily intake of 405 kcal/day in the form of 3 sachets, or 470 kcal/day if one of the sachets was replaced by a flapjack. For men, the diet was provided in the form of 2 sachets providing 540 kcal/day, or one sachet and two flapjacks increasing the daily calories to 670 kcal/day. The contents of the sachets were mixed with approximately 250 ml of water, forming a liquid drink, soup or mousse and patients were advised to drink 2-4 l of water throughout the day. Beverages such as tea or coffee, with no added milk or sugar, were permitted in order to prevent symptoms of caffeine withdrawal. However, other beverages, including milk or alcohol were not permitted whilst consuming the VLCD. Consequently, a large calorie gap was created between energy intake and expenditure, enabling rapid weight loss in all patients. Patients on the VLCD developed mild ketosis, as a result of the oxidation of fatty acids released from adipose tissue by the liver, which caused a substantial blunting of hunger.

Patients were required to attend weekly for maximal compliance with the diet and in order to monitor progress. Ketosis was used as an index of compliance and urinary ketones were measured at each visit using Ketostix (Bayer Diagnostics, Bayer Plc., Basingstoke, UK). Diabetic patients commencing on the VLCD were advised to stop taking oral hypoglycaemic agents, or to halve their insulin dosage on day one of the diet. Hypotensive and hypolipidaemic treatments were adjusted after one month if necessary. Each patient on the VLCD set a target for weight loss and received counselling during the period of rapid weight loss and during the period of weight maintenance after stopping the VLCD.

Group discussions were held weekly, providing additional encouragement and support. After stopping the VLCD, patients were warned to expect an initial rapid increase in weight, due to the repletion of glycogen stores. In order to keep this initial weight gain to a minimum, the patients were provided with a refeeding strategy; a brief outline of this strategy is described in Appendix 4.

Patients who had transferred from the VLCD to weight maintenance diets were encouraged to attend the group discussions at least monthly. A video and printed information, describing the physiology of weight loss and strategies for the transition from the VLCD to low-fat foods, for weight maintenance, were provided as part of the Lipotrim programme for educational purposes. Demonstrations of low-fat cooking techniques were also held and books containing additional information on weight maintenance strategies were available. Repeat courses of the VLCD were available to the patients, if necessary, for the duration of the 3 year study.

The VLCD sessions were conducted by two nurses and took place in parallel with the sessions for the ICD group. A physician and a trained counsellor were available to both groups. Each session was approximately 1.5-2 hours long and took place in adjoining medical-ward day rooms. The sessions took place on a weekly basis for two years and thereafter on a monthly basis for the final year.

7.3.2 Intensive conventional dietetic therapy group

The sessions were conducted by two dieticians. Five day food records were used to provide information on habitual dietary intake and to enable erratic eating patterns to be identified. Each subject set a target weight, completed a 5 day food record and then received individual dietetic assessment and advice tailored to their social position, physical activity and nutrient requirement. The dietary advice was based on the recommendations of the Nutrition Subcommittee of the British Diabetic Association (1992). The emphasis of the dietetic advice was to reinforce **healthy eating principles** by encouraging the patients to eat regular meals, increase their intake of fresh fruit and vegetables, lower their fat and sugar intake and increase their intake of complex carbohydrates (thereby reducing the daily energy intake by approximately 300 kcal/day), rather than providing prescriptive dietary therapy and low-calorie diets (800-1200 kcal/day). Five day food records were completed every 6-8 weeks and the dieticians were available for consultation each week if the patients required further individual advice. Group discussions took place weekly, covering various nutritional and behavioural topics. Gentle aerobic exercises were performed to music, for 30-45 minutes, during the sessions in order to encourage the patients to increase their levels of exercise. Additional input to the groups was provided by physiotherapists and a health promotion officer, to encourage the patients to adopt more active lifestyles. The sessions were held weekly and patients were encouraged to attend for as long as was necessary.

7.4   Methods

The effect of the dietary interventions on anthropometric measurements (weight loss, BMI, waist / hip ratios, blood pressure), metabolic factors (serum triglycerides, cholesterol and HDL cholesterol), glycaemic control (glucose, fructosamine), markers of free radical activity (MDA and conjugated dienes) and antioxidants (vitamins A, C, E and uric acid) were investigated.

Body weight was recorded at each visit. Blood pressure measurements were taken in the sitting position at baseline, 1, 3, 6 and 12 months, using a mercury sphygmomanometer. Waist and hip measurements were taken to include the largest circumferences possible at baseline 3, 6 and 12 months.

The patients were asked to stop taking any form of dietary supplements 1-2 weeks before commencing on the dietary interventions. Blood samples were taken 2-3 hours post prandially, during the weekly visits before commencing on either dietary regime (baseline) and then repeated at 1, 3, 6 and 12 months. Blood samples were collected into tubes containing separator gel for the analysis of serum cholesterol, triglycerides, fructosamine, uric acid and HDL-cholesterol. Blood for the analysis of plasma glucose was collected into tubes containing sodium fluoride. The above analyses were performed at the Chemical Pathology Department at Torbay Hospital as described previously (section 6.3, page 129). High-density lipoprotein cholesterol was measured after phosphoric acid and magnesium ion precipitation of chylomicrons, VLDL and LDL, using an enzymatic-colorimetric kit adapted for the Hitachi 717 analyser (Boehringer, Mannheim, Germany). Serum fructosamine measurement was based upon the reduction with nitroblue tetrazolium, standardized with glycosylated polylysine, using a Hitachi 717 analyser. Blood samples were collected into 10 ml tubes containing EDTA for the analysis of plasma vitamins A, C, E, MDA the conjugated diene isomer of linoleic acid and its molar ratio to linoleic acid. Plasma was separated and frozen at -70°C within two hours of collection for these analyses. An overview of the study is shown in Figure 7.1.

Patients: Obese type II diabetic patients and obese non-diabetic subjects with no severe intercurrent illnesses other than hyperlipidaemia, hypertension or gout.

Age: 25 - 70 years   Waist / hip ratio: > 0.8 Females
BMI: > 30   > 1.0 Males

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↓

52   Patient self-selection
                            of dietary regime

VLCD   ICD

N   Dm   N   Dm   N — non-diabetic subjects
($n = 18$) ($n = 15$) ($n = 14$) ($n = 5$)   Dm — diabetic subjects
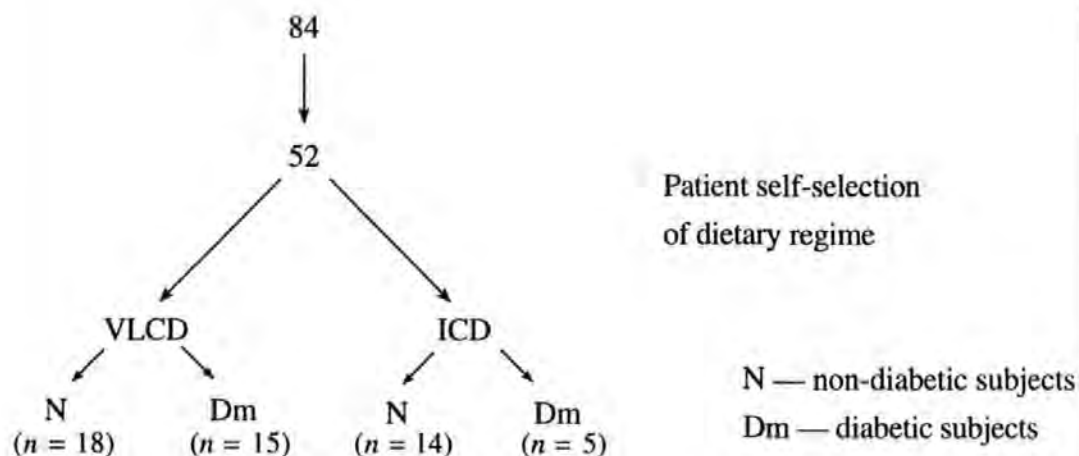
H_0: No difference between diabetic and non-diabetic patients on the VLCD or ICD regimes.

H_A: There is a difference between diabetic and non-diabetic patients on the dietary regimes.

Investigations: Baseline, 1, 3, 6 and 12 months.

Anthropometric:	**Biochemical:**	**Indices of oxidative stress:**
Weight	Glucose	Vitamins A, C and E
BMI	Fructosamine	Uric acid
Waist / hip ratio	Triglycerides	Lipid peroxides
Blood pressure	Cholesterol (HDL)	(MDA and conjugated dienes)

Figure 7.1 *Overview of the VLCD and ICD study.*

Statistical analyses

The normality of the data were checked by plotting normal scores and by using the Shapiro-Wilk test (Rees 1995). Triglyceride values were log transformed to improve normality, although the actual values are presented in the tables. Differences within and between the groups were assessed by analysis of variance. Where the F-test showed a significance, further analysis was carried out using the Tukey test and also by using paired and unpaired t tests. Correlations were carried out using Pearson's product moment correlation coefficient. The results are presented as mean ± SD unless stated otherwise. A p value < 0.05 was considered to be significant. Statistical analyses were performed by using Excel 7.0 and Minitab 10.51 Xtra software programmes.

7.5   Results

The results presented are the changes during the period of acute weight loss (0-6 months) and during the period of weight maintenance (12 month data).

The patients were not randomized to the two dietary interventions, but chose which treatment they would prefer, therefore, a direct comparison between the two treatments in order to establish which treatment was the most effective long-term weight loss therapy cannot be made. However, by allowing the patients to choose their specific dietary regimes, greater patient compliance was ensured. This was of particular importance in the VLCD programme, since good patient compliance was necessary in order to ensure that the VLCD would be used as a total food replacement; any biochemical changes that were taking place would then be clearly established. Consequently, the morale of the patients was high and the patient groups were highly motivated at the beginning of the dietary interventions. However, due to non-compliance, only 5 patients were recruited into the ICD non-diabetic control group and their data has been omitted from the main analysis.

In the VLCD group, oral hypoglycaemic agents and diuretics were stopped on day one and insulin dosage halved in patients requiring insulin therapy and then stopped completely after one week. After one month on the VLCD, only one of the six insulin-treated patients required insulin as part of their therapy. Anti-hypertensive treatments were discontinued at one month in all but one of the diabetic subjects on the VLCD. Three of the insulin treated diabetic patients in the ICD group reduced their insulin dosages during the initial six months, but no other adjustments in treatments were required.

The patients were enthusiastic, although initially apprehensive about consuming a total food replacement diet. However, after the first 3 days on the VLCD, the majority of the patients experienced little difficulty in consuming the diet. The development of mild ketosis promoted a feeling of 'well-being' and prevented hunger, whilst rapid weight loss encouraged the patients to continue with the diet. Target weights were reached after a duration of 4 months on the VLCD (18 ± 4 weeks, on average, for the non-diabetic subjects

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and 16 ± 7 weeks for the diabetic subjects). The VLCD was used by 7 patients for 1-2 months, to assist in the transition to normal food and for weight maintenance, by using one Lipotrim sachet as a substitute for one daily meal. All patients had reverted to normal food by 6 months, except for one diabetic patient who remained on the VLCD for a period of 9 months.

The attendance rates of the patients in the VLCD groups were high during the acute weight loss phase (70-90%), thereafter the attendance rates declined and were between 45-55% during the period of weight maintenance. In contrast, the attendance rates of the patients in the ICD group remained stable throughout the study period (50-60%). All patients, however, attended their respective review clinics.

Adverse reactions

One diabetic patient did not reduce their insulin dosage sufficiently during the first week of the VLCD and suffered a severe hypoglycaemic episode. The insulin dosage was lowered further as a result of this outcome. One female non-diabetic patient developed alopecia whilst on the VLCD and several patients suffered from severe headaches during the first two weeks on the VLCD, due to insufficient fluid intake. One female non-diabetic patient experienced elevations in hepatic enzymes as a result of rapidly stopping and then restarting the VLCD. One diabetic male patient in the ICD group suffered an acute myocardial infarction during the first month of the study. In the VLCD groups, one non-diabetic male patient and one female diabetic patient suffered acute non-fatal myocardial infarctions after 6 months.

Baseline comparisons

The groups were well matched at the beginning of the dietary interventions. No significant differences were found in anthropometric variables, although diabetic patients in the ICD group were lighter (7 kg) than the patients in the two VLCD groups and systolic blood pressures were slightly higher (9 mm Hg) in both diabetic groups compared with the non-diabetic group. As expected, plasma glucose and fructosamine concentrations were significantly higher in the diabetic patients than in the non-diabetic patients ($p < 0.001$ for all comparisons). Interestingly, serum triglyceride, HDL cholesterol levels were similar in all of the groups.

There were no significant differences between the groups in relation to the plasma antioxidants, although plasma ascorbate concentrations were slightly lower in the diabetic patients. Plasma MDA concentrations were slightly higher in the diabetic patients compared with the non-diabetic patients, although the differences were not significant. However, plasma MDA concentrations were significantly higher in both diabetic and non-diabetic groups compared with a healthy non-obese control group ($p < 0.001$).

The number of smokers in the study was small — two patients in the VLCD control group and one diabetic patient in the ICD group — and therefore their data was included in the main analyses. Two diabetic patients in the VLCD group were newly diagnosed with type II diabetes. The duration of diabetes in the remaining patients in both groups was between 5 and 10 years.

7.5.1 Anthropometric measurements

A summary of the overall changes in anthropometric measurements is shown in Table 7.2.

(i) Weight

The changes in weight in the treatment groups are shown in Table 7.3 and Figure 7.2. Rapid weight losses occurred in the two VLCD groups with significant reductions at 1 and 3 months ($p < 0.0001$), which were maintained at 6 and 12 months ($p < 0.007$). The mean weight losses at 1, 3 and 6 months in the VLCD non-diabetic group were 10.5, 20.7 and 22.4 kg respectively. At the 12 month follow-up, 11 of the patients had regained more than 5 kg, resulting in a mean net weight loss of 15.5 kg at 12 months. In comparison, lower weight losses were achieved by the diabetic patients on the VLCD, with reductions of 9.2, 15.2, 15.2 kg at 1, 3 and 6 months respectively. The diabetic patients also regained a small amount of weight (with only 4 patients regaining more than 5 kg), so that the net loss at 12 months was 13.5 kg in this group. Thus, the weight loss in the non-diabetic VLCD group was significantly greater than in the diabetic VLCD group at 3 months ($p = 0.04$, Table 7.3).

One diabetic patient remained on the VLCD for a period of 9 months, losing a remarkable 70 kg of weight by 12 months. When the data were re-analysed without this patient, the significant differences that were found within the diabetic VLCD group remained. However, when the two VLCD groups were compared, the non-diabetic patients were found to have lost significantly more weight than the diabetic patients at 6 and 12 months ($p = 0.0007$ and $p = 0.04$ respectively, Table 7.3). No other differences in other anthropometric measurements or serum biochemistry were found when this patient's data were removed from the analyses.

The mean weight losses in the ICD group were 1.7, 3.4, 3.4 and 2.3 kg at 1, 3, 6, and 12 months respectively and reached statistical significance at 1, 3 and 6 months ($p < 0.02$). Two patients in the ICD group gained weight during the 12 months of the study and were regarded as treatment failures. When the data were re-analysed without these patients, significant reductions in weight were found at all of the follow-up times ($p < 0.01$) and the mean weight losses were 2, 4.2, 4.2 and 3.7 kg at 1, 3, 6 and 12 months respectively. Comparisons of the two diabetic groups revealed that the diabetic patients had lost significantly more weight on the VLCD than the ICD therapy at 1, 3, 6 and 12 months (Table 7.3).

Table 7.2 *Changes in anthropometric measurements from baseline to 12 months.*

		PATIENT GROUPS		
		VLCD Non-diabetic Subjects (n = 18)	VLCD Diabetic Subjects (n = 15)	ICD Diabetic Subjects (n = 14)
BMI	Baseline	38 ± 4	38 ± 10	36 ± 5
(kg/m²)	1 month	34 ± 4 [a]	34 ± 9 [a]	35 ± 5 [d]
	3 months	30 ± 4 [a]	32 ± 8 [a]	35 ± 6 [d]
	6 months	30 ± 4 [a]	32 ± 7 [b]	35 ± 6 [d]
	12 months	32 ± 4 [a]	33 ± 9 [c]	35 ± 6
Waist	Baseline	114 ± 13	119 ± 20	111 ± 11
(cm)	3 months	95 ± 9 [a]	102 ± 13 [a]	109 ± 11
	6 months	95 ± 10 [a]	103 ± 14 [b]	110 ± 12
	12 months	100 ± 12 [b]	104 ± 18 [b]	111 ± 12
Waist /	Baseline	0.94 ± 0.1	0.98 ± 0.09	0.91 ± 0.08
Hip	3 months	0.86 ± 0.07 [c]	0.92 ± 0.08 [d]	0.93 ± 0.07
Ratio	6 months	0.88 ± 0.05 [d]	0.93 ± 0.07	0.91 ± 0.08
	12 months	0.88 ± 0.08	0.93 ± 0.08 [d]	0.92 ± 0.07
Systolic	Baseline	131 ± 21	140 ± 18	141 ± 20
Blood	1 month	115 ± 14 [b]	129 ± 14 [d]	139 ± 17
Pressure	3 months	112 ± 11 [b]	134 ± 18	134 ± 19
(mm Hg)	6 months	120 ± 18 [d]	137 ± 14	134 ± 17
	12 months	118 ± 17 [c]	143 ± 19	138 ± 19
Diastolic	Baseline	82 ± 12	77 ± 11	85 ± 12
Blood	1 month	73 ± 11 [c]	74 ± 12	82 ± 11 [d]
Pressure	3 months	69 ± 10 [b]	74 ± 11	78 ± 8 [d]
(mm Hg)	6 months	68 ± 8 [a]	78 ± 10	71 ± 9 [b]
	12 months	72 ± 9 [b]	81 ± 11	78 ± 10

Means were significantly different from the baseline values, within the groups: [a] $p < 0.0001$; [b] $p < 0.001$; [c] $p < 0.01$; [d] $p < 0.05$.
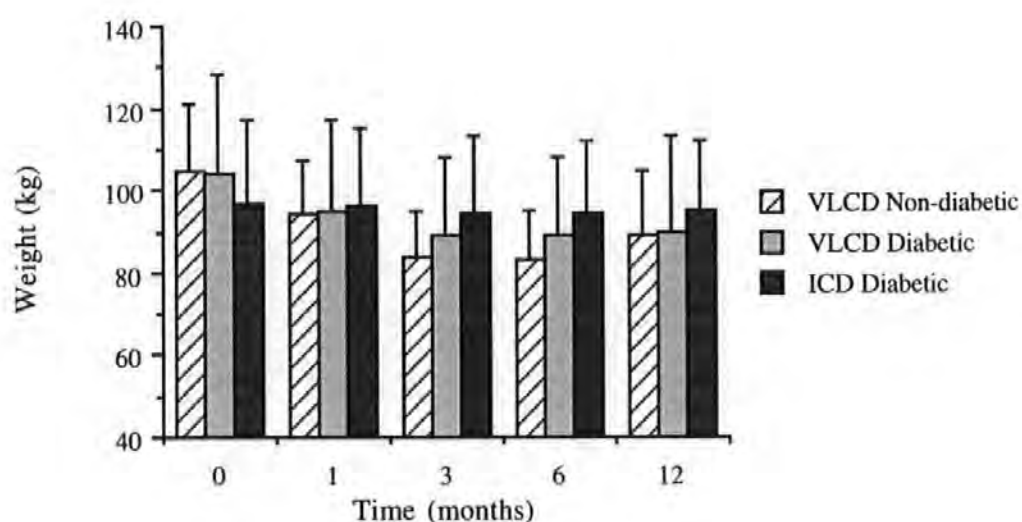
Figure 7.2 *Changes in weight during the first year of the study.*

Table 7.3 *Mean weight losses on the dietary interventions.*

Group	Baseline Weight (kg)	Weight change at			
		1 Month	3 Months	6 Months	12 Months
VLCD Non-Diabetic (*n* = 18)	105 ± 16	-10.5 ± 4	-20.7 ± 7 *a*	-22.4 ± 7	-15.5 ± 10
VLCD Diabetic (*n* = 15)	104 ± 24	-9.2 ± 3 *b*	-15.2 ± 7 *b*	-15.2 ± 13 *c* * (-12.4 ± 7) *e*	-13.5 ± 17 *d* (- 9.5 ± 6) *f*
ICD Diabetic (*n* = 14)	97 ± 20	-1.7 ± 2	-3.4 ± 4	-3.4 ± 3	-2.3 ± 5

Minus sign indicates the mean ± SD decrease in the group weights at the follow-up intervals.

VLCD non-diabetic compared with VLCD diabetic patients: *a* *p* = 0.04.
VLCD diabetic compared with ICD diabetic patients: *b* *p* = 0.00001, *c* *p* = 0.004, *d* *p* = 0.02.
*The data were re-analysed without one diabetic patient who remained on the VLCD for > 6 months: VLCD non-diabetic compared with VLCD diabetic patients (*n* = 14): *e* *p* = 0.0007, *f* *p* = 0.04.

(ii) BMI

The reduction in weight was accompanied by significant reductions in BMI in the two VLCD groups at all of the follow-up times (Table 7.2 and Figure 7.3). In the ICD group, significant reductions in BMI were observed at 1, 3 and 6 months ($p < 0.05$).

The percentage of patients attaining a BMI $\leq$ 30 reached a maximum at the end of the acute weight-loss phase in the VLCD non-diabetic group (67% at 6 months), whereas in the VLCD diabetic group 9 of the 15 patients (60%) achieved a BMI $\leq$ 30 after 12 months (Table 7.4). In contrast, only 14% of the ICD diabetic patients achieved a BMI of 30 or below at 6 months. When the groups were compared, the reduction in BMI was found to be significantly greater in the non-diabetic subjects than in the diabetic subjects on the VLCD at 3 months ($p = 0.05$). The reduction in BMI was also significantly greater in the VLCD diabetic patients compared with the ICD diabetic patients at all of the follow-up times ($p < 0.02$).

(iii) Waist / hip ratio

The changes in waist measurements and waist / hip ratios are shown in Figures 7.4 and 7.5. Waist circumferences were significantly reduced in the two VLCD groups at all of the follow up times ($p < 0.001$). Waist / hip ratios were significantly reduced in the VLCD non-diabetic subjects at 3 and 6 months ($p < 0.05$). In the VLCD diabetic group, significant reductions in waist / hip ratios were observed at 3 months and maintained 12 months ($p < 0.05$). The reductions in waist and waist / hip measurements were greater in the non-diabetic subjects on the VLCD than in the diabetic subjects on the VLCD at 3 and 6 months ($p < 0.04$).

In contrast, no significant changes in waist measurements or waist / hip ratios were observed in the ICD group during the 12 months of follow-up. Thus, the diabetic patients on the VLCD achieved significantly greater reductions in waist measurements than the diabetic patients on the ICD therapy at all of the follow-up times ($p < 0.007$).

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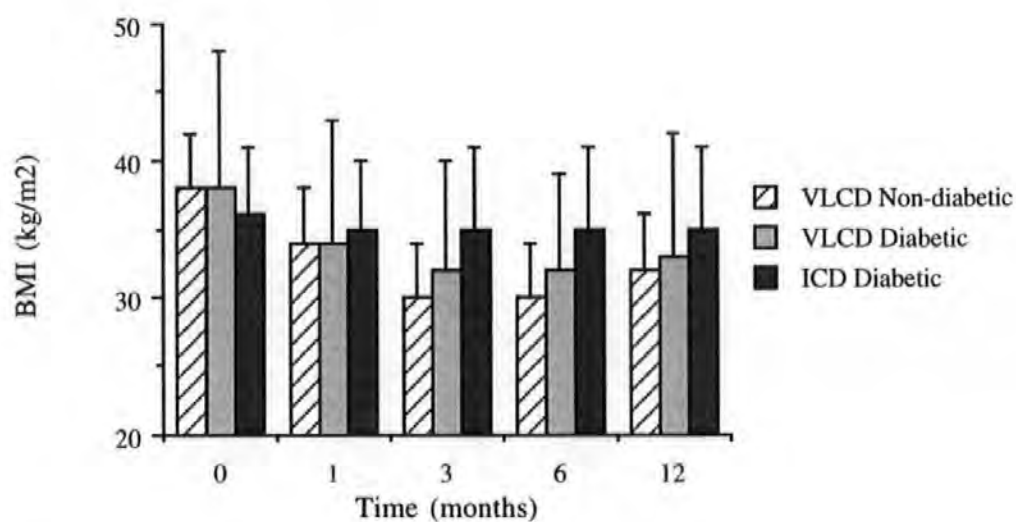
Figure 7.3 *Changes in BMI during the first year of the study.*

Table 7.4 *Percentage of patients achieving a BMI ≤ 30.*

	VLCD Non-Diabetic		**VLCD-Diabetic**		**ICD-Diabetic**	
BMI	≤ 30 (%)	≥35	≤ 30	≥35	≤ 30	≥ 35
Baseline	0	72	20	47	0	7
1 Month	22	44	40	27	7	7
3 Months	56	17	40	27	7	7
6 Months	67	11	47	27	14	7
12 Months	33	28	60	27	7	7
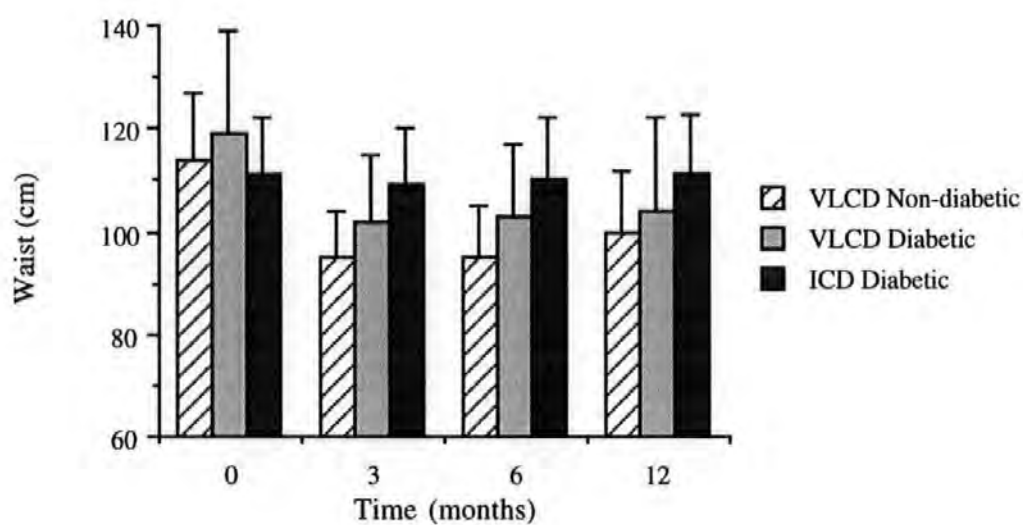
Figure 7.4 *Changes in waist circumferences.*
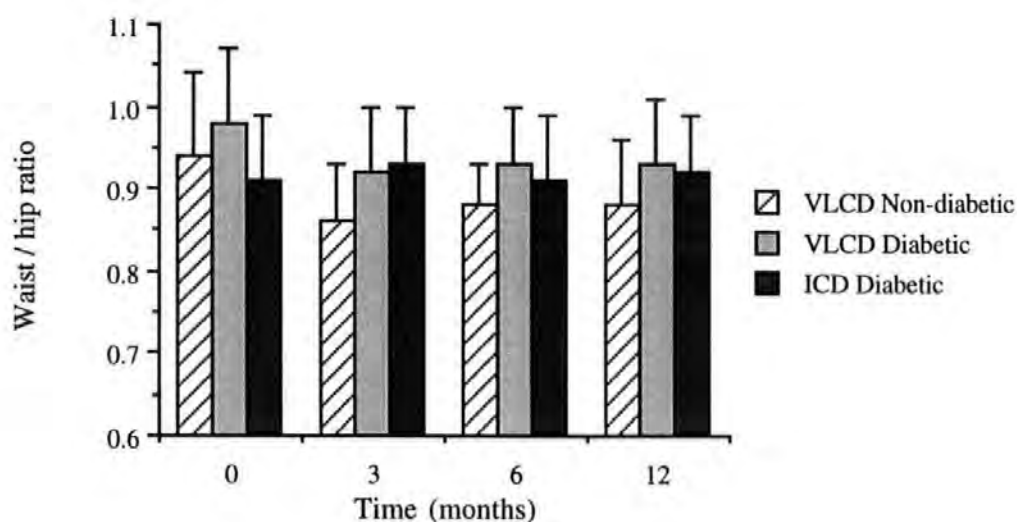
Figure 7.5 *Changes in waist / hip ratios.*

(iv) Blood pressure

Figures 7.6 and 7.7 show the changes in blood pressure in the patients groups. Systolic blood pressures decreased significantly in the VLCD non-diabetic group during the acute weight loss phase and were lowest at 3 months, with a mean reduction of 19 mm Hg at this time ($p < 0.001$). Significant reductions were maintained at 6 and 12 months and systolic blood pressures were on average 10 mm Hg lower at the 12 month follow-up compared with the baseline values ($p < 0.01$). In contrast, a significant decrease in systolic blood pressure was only observed at 1 month in the diabetic patients on the VLCD, with a mean maximum reduction of 10 mm Hg at this time ($p < 0.05$). Thereafter, systolic blood pressures returned to baseline values and were on average 3 mm Hg higher at the 12 month follow-up than at the start of the VLCD in the diabetic patients. In the ICD group, systolic blood pressures remained stable during the 12 months of follow-up and showed no significant changes.

Diastolic blood pressures also decreased significantly at all of the follow-up times in the non-diabetic VLCD group ($p < 0.01$). A maximum average reduction of 13 mm Hg was found after 6 months which was maintained at 12 months. No significant reductions in diastolic blood pressure were observed during the acute weight loss phase or after 12 months in the diabetic patients on the VLCD. In the ICD group, significant reductions in diastolic blood pressure were observed at 1, 3 and 6 months, with a mean reduction of 14 mm Hg at 6 months ($p < 0.001$). However, at the 12 month follow-up the mean reduction in diastolic blood pressure was 8 mm Hg and did not quite reach statistical significance ($p = 0.058$).

When the groups were compared, systolic blood pressures were found to be significantly lower in the non-diabetic VLCD group at 1, 3, 6 and 12 months ($p < 0.009$) compared with the diabetic VLCD group. Diastolic blood pressures were also lower in the non-diabetic VLCD group compared with the diabetic VLCD group at 6 months ($p = 0.005$) and at 12 months ($p = 0.01$). Significantly greater reductions in diastolic blood pressures were also observed in the ICD diabetic patients compared with the diabetic patients on the VLCD at 6 months ($p = 0.001$) and at 12 months ($p = 0.02$).
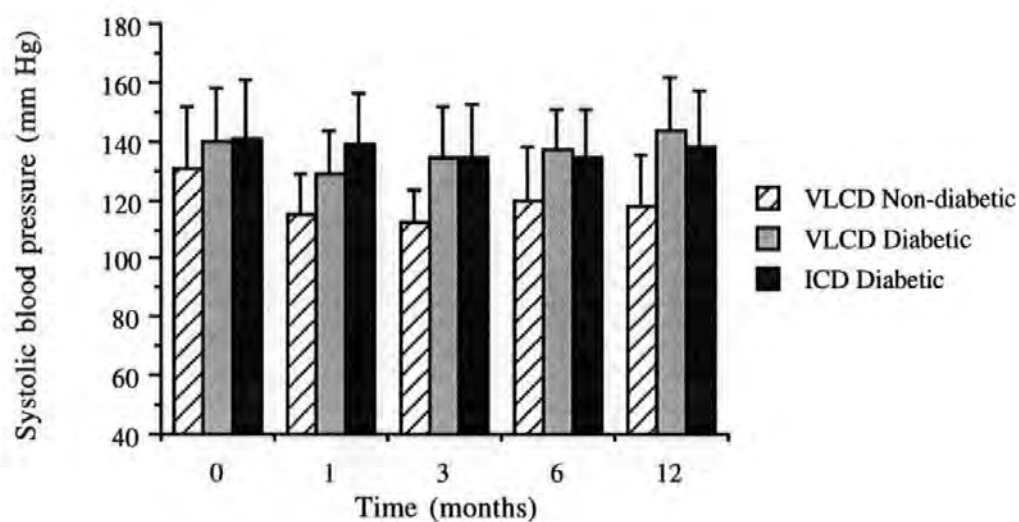
Figure 7.6 *Changes in systolic blood pressure.*
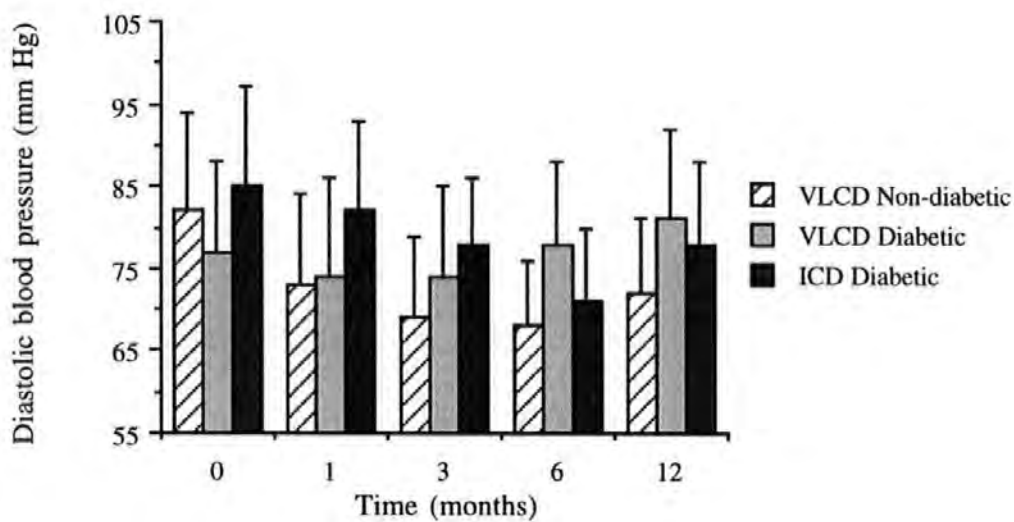
Figure 7.7 *Changes in diastolic blood pressure.*

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7.5.2 Serum biochemistry

Table 7.5 summarizes the overall changes in metabolic control during the first year of the study.

(i) Glycaemic control

Post-prandial plasma glucose concentrations fell from 12.4 ± 3 mmol/l to 8.1 mmol/l ($p = 0.0004$) after 1 month of VLCD in the diabetic patients. The decrease in plasma glucose was accompanied by a significant reduction in serum fructosamine at 1 month ($p < 0.001$) which was maintained at 3 months ($p < 0.05$) in this group. Thereafter, serum glucose and fructosamine concentrations increased and had returned to baseline values after 6 and 12 months (Figures 7.8 and 7.9).

In comparison, plasma glucose concentrations showed a slight increase at 3 months in the ICD diabetic group, but serum fructosamine concentrations remained stable throughout the 12 months of follow-up (Figures 7.8 and 7.9). Glucose and fructosamine concentrations were similar in the two diabetic groups at baseline, but were significantly lower in the VLCD group compared to the ICD group at 1 month ($p = 0.03$ and $p = 0.01$ respectively). Indices of glycaemic control were strikingly stable in the non-diabetic subjects during the acute weight loss phase and weight maintenance (Figures 7.8 and 7.9), and remained significantly lower than the diabetic groups at all of the follow-up points ($p < 0.005$).

Table 7.5 *Summary of the changes in serum biochemistry.*

		PATIENT GROUPS		
		VLCD Non-diabetic	VLCD Diabetic	ICD Diabetic
Plasma	Baseline	5.1 ± 0.8	12.4 ± 4.7	13.1 ± 5.5
Glucose	1 month	4.7 ± 0.8	8.1 ± 3.1 ^{b}	13.1 ± 7.1
(mmol/l)	3 months	5.0 ± 0.4	10.6 ± 5.8	16.8 ± 8.8
	6 months	5.0 ± 0.5	11.2 ± 5.5	12.4 ± 5.3
	12 months	5.0 ± 0.4	11.7 ± 7.7	13.3 ± 5.6
Serum	Baseline	236 ± 11	345 ± 81	386 ± 99
Fructosamine	1 month	231 ± 15	274 ± 45 ^{c}	372 ± 118
(mmol/l)	3 months	229 ± 17	298 ± 69 ^{e}	384 ± 142
	6 months	235 ± 19	325 ± 91	369 ± 121
	12 months	232 ± 20	345 ± 94	380 ± 106
Serum	Baseline	2.5 (2.1 - 3.7) *	2.7 (1.9 - 5.8)	2.2 (1.5 - 3.7)
Triglycerides	1 month	1.3 (1.1 - 1.6) ^{a}	1.5 (1.1 - 1.8) ^{e}	1.9 (1.6 - 3.5)
(mmol/l)	3 months	1.5 (1.1 - 1.8) ^{a}	1.6 (1.0 - 2.4) ^{e}	2.5 (1.7 - 3.4)
	6 months	1.5 (1.2 - 1.8) ^{a}	1.8 (1.1 - 3.4) ^{e}	2.1 (1.8 - 2.5)
	12 months	1.8 (1.5 - 2.1) ^{a}	1.8 (1.1 - 4.3)	2.3 (1.6 - 4.7)
Serum	Baseline	6.7 ± 1.3	6.8 ± 1.2	5.9 ± 1.3
Cholesterol	1 month	4.6 ± 1.0 ^{a}	5.1 ± 1.2 ^{a}	6.2 ± 1.4
(mmol/l)	3 months	5.6 ± 1.0 ^{d}	5.7 ± 1.0 ^{d}	6.1 ± 1.8
	6 months	6.1 ± 1.1 ^{e}	5.8 ± 1.1 ^{d}	6.0 ± 1.6
	12 months	6.2 ± 1.0	6.3 ± 1.6	6.2 ± 1.4
HDL / Total	Baseline	0.18 ± 0.06	0.17 ± 0.06	0.21 ± 0.08
Cholesterol	1 month	0.22 ± 0.07 ^{d}	0.20 ± 0.07	0.20 ± 0.08
Ratio	3 months	0.21 ± 0.08 ^{e}	0.20 ± 0.07	0.21 ± 0.09
	6 months	0.22 ± 0.07 ^{d}	0.21 ± 0.07 ^{d}	0.19 ± 0.06
	12 months	0.23 ± 0.05 ^{d}	0.23 ± 0.08 ^{b}	0.21 ± 0.08

* Median (range). Means were significantly different from the baseline values, within the groups: ^{a} $p < 0.0001$; ^{b} $p < 0.0004$; ^{c} $p < 0.001$; ^{d} $p < 0.01$; ^{e} $p < 0.05$.
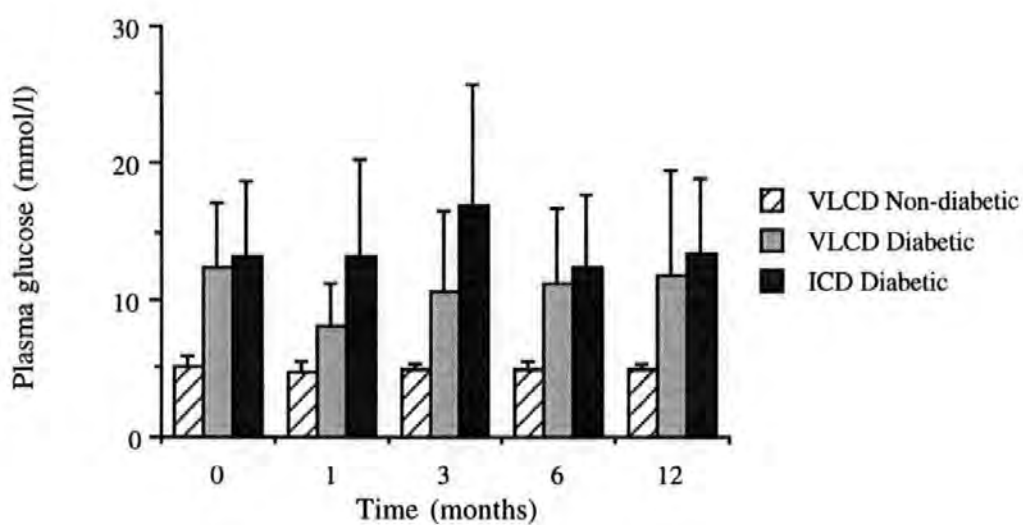
Figure 7.8 *Changes in plasma glucose concentrations.*
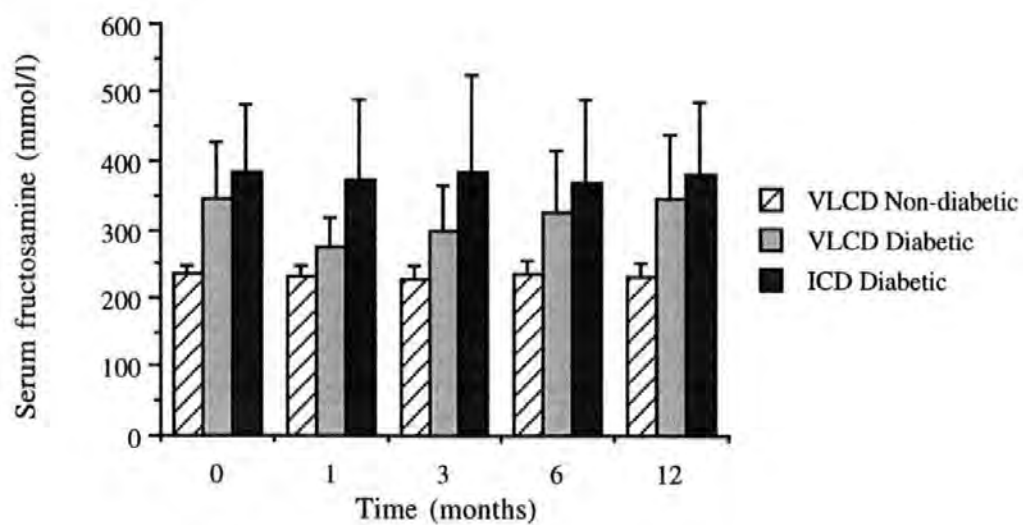
Figure 7.9 *Changes in serum fructosamine.*

(ii) Serum lipids

Changes in serum lipids are shown in Figures 7.10 and 7.11. Serum triglycerides were significantly reduced in the non-diabetic subjects on the VLCD at 1 and 3 months and the improvements were maintained at 6 and 12 months ($p < 0.0001$). Significant reductions were also found in the diabetic patients on the VLCD at 1, 3 and 6 months ($p < 0.05$), but the improvements were not sustained at 12 months, although triglyceride levels were on average 0.9 mmol/l lower than at baseline.

Serum cholesterol levels were significantly reduced at 1 month in both VLCD groups ($p < 0.0001$) and were also significantly lower than the ICD group at this time ($p < 0.04$). The reductions in cholesterol were maintained at 3 and 6 months (Table 7.5). At 12 months, cholesterol levels were on average 0.5 mmol/l and 0.4 mmol/l lower than the baseline levels in the non-diabetic and diabetic VLCD patients respectively. In comparison, serum triglyceride and cholesterol levels showed no significant changes in the ICD group during the 12 months of follow-up. At 12 months, triglyceride levels were on average 0.7 mmol/l higher and cholesterol levels were 0.3 mmol/l higher than at the start of the study.

Changes in HDL levels and in the HDL / total cholesterol ratio are shown in Figures 7.12 and 7.13. High density lipoprotein cholesterol concentrations showed a slight decrease in both VLCD groups at 1 month, but increased thereafter and were significantly higher at 6 and 12 months in the non-diabetic subjects ($p < 0.005$) and after 12 months in the diabetic subjects ($p < 0.001$). Significant improvements in the HDL / total cholesterol ratio were found in the non-diabetic patients after 1 month and these were maintained at 3, 6 and 12 months ($p < 0.05$). A significant increase in the ratio was also found in the VLCD diabetic group at 6 months ($p < 0.01$), which was sustained at 12 months ($p < 0.0004$). Interestingly, a significant reduction in HDL cholesterol was observed in the ICD group at 6 months ($p = 0.01$), but the HDL / total cholesterol ratios remained stable during the follow-up period and were not significantly different from those in the VLCD groups. Positive correlations were found between the change in weight and the change in cholesterol at 1 ($p < 0.001$) and 3 months ($p < 0.01$) and the change in triglycerides at 1, 3 and 6 months ($p < 0.01$).
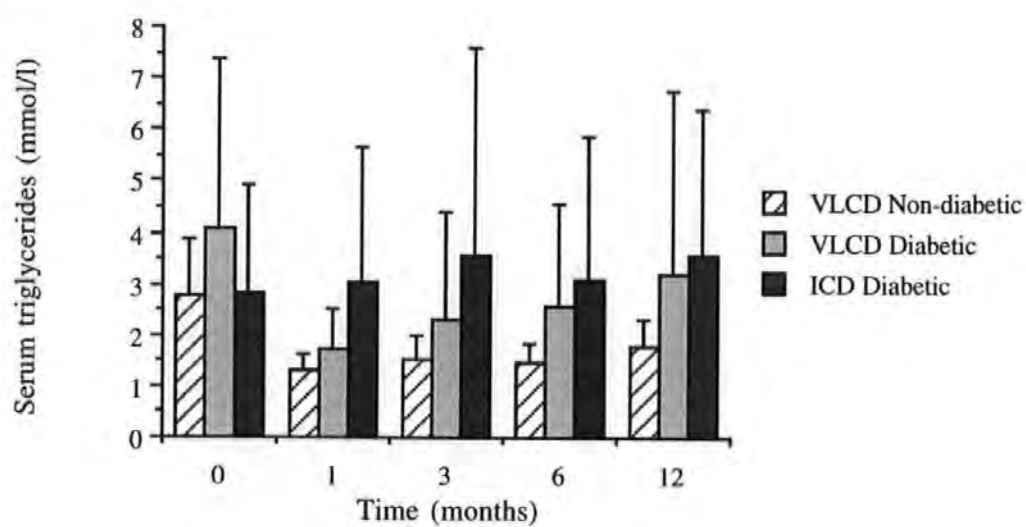
Figure 7.10 *Changes in serum triglycerides.*
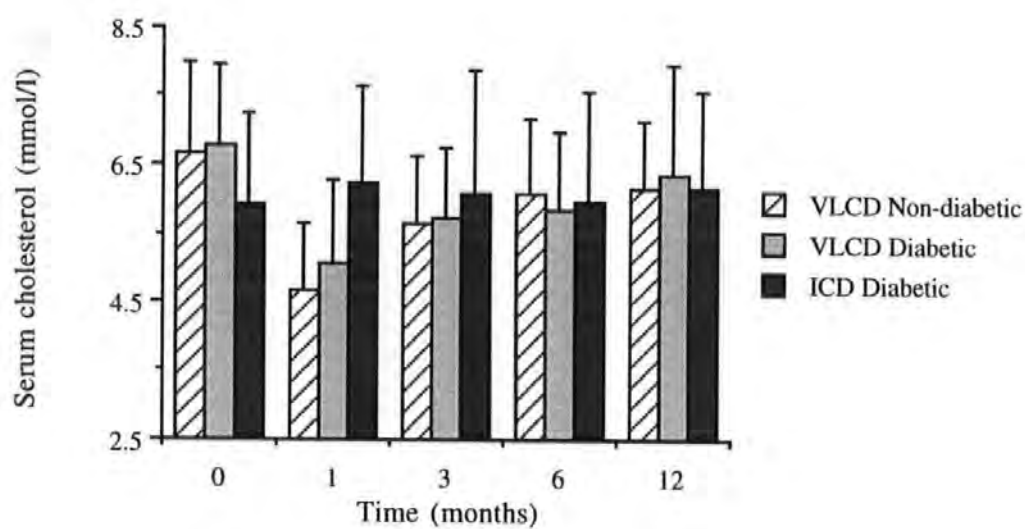
Figure 7.11 *Changes in serum cholesterol.*
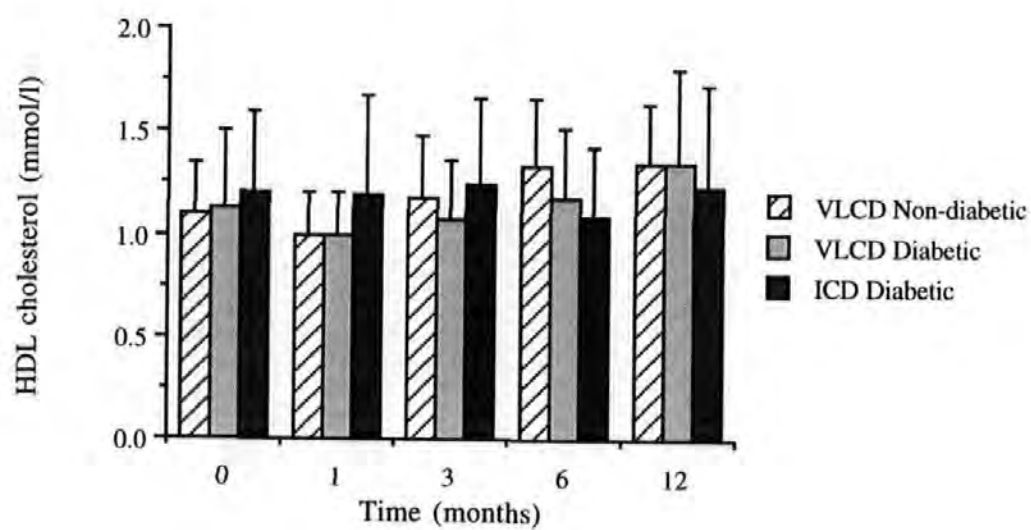
Figure 7.12 *Changes in serum HDL cholesterol concentrations.*
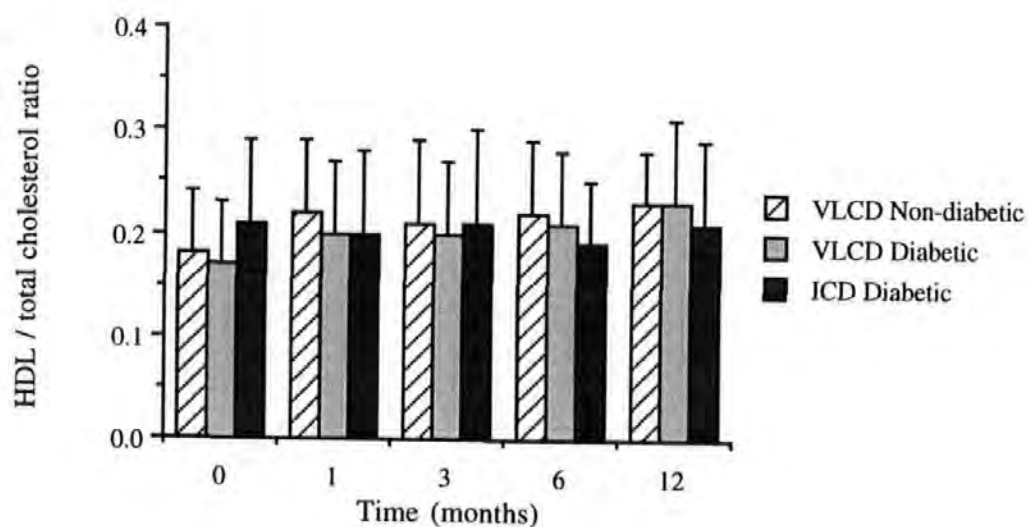
Figure 7.13 *Changes in serum HDL / total cholesterol ratios.*

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7.5.3 Plasma antioxidants

Table 7.6 summarizes the changes in plasma antioxidants during the 12 months of follow-up.

(i) Lipid-soluble antioxidants

Figures 7.14 - 7.16 show the changes in the lipid-soluble antioxidants in plasma in the patient groups during the follow-up period. Plasma retinol concentrations were significantly reduced at 1 month in both VLCD groups ($p < 0.02$) and remained lower than baseline values after 3 months in the non-diabetic subjects ($p < 0.03$). Plasma α-tocopherol concentrations were also significantly reduced in both VLCD groups at 1 month ($p < 0.007$) and remained below baseline levels at 3 months in the diabetic patients ($p < 0.05$) and at 6 and 12 months in the non-diabetic patients ($p < 0.03$). However, the α-tocopherol / triglyceride + cholesterol (lipid) ratios increased gradually in both groups, although the differences did not reach statistical significance (Figure 7.16).

In the ICD group, retinol and α-tocopherol concentrations remained stable throughout the follow-up period. However, the α-tocopherol / lipid ratios showed a gradual decline and were significantly lower than the baseline values after 12 months ($p < 0.05$) in this group. Plasma α-tocopherol / lipid ratios were also significantly lower in the ICD patients compared with the VLCD diabetic patients at 6 months ($p < 0.05$). No differences in lipid-soluble antioxidants were found between the diabetic patients and the non-diabetic patients on the VLCD.

Table 7.6 *Summary of the changes in plasma antioxidants.*

		PATIENT GROUPS		
		VLCD Non-diabetic	VLCD Diabetic	ICD Diabetic
Retinol	Baseline	1.9 ± 0.4	1.9 ± 0.5	2.1 ± 0.6
(μmol/l)	1 month	1.3 ± 0.3 [a]	1.6 ± 0.7 [b]	2.3 ± 0.8
	3 months	1.7 ± 0.5 [c]	1.8 ± 0.8	2.1 ± 0.6
	6 months	2.0 ± 0.4	2.1 ± 0.8	2.1 ± 0.6
	12 months	2.0 ± 0.5	2.0 ± 0.8	2.2 ± 0.6
α-Tocopherol	Baseline	35 ± 11	39 ± 14	32 ± 14
(μmol/l)	1 month	22 ± 4 [a]	27 ± 9 [e]	36 ± 18
	3 months	30 ± 12	32 ± 12 [d]	35 ± 23
	6 months	29 ± 6 [e]	34 ± 12	30 ± 14
	12 months	29 ± 6 [c]	36 ± 19	32 ± 13
α-Tocopherol /	Baseline	3.7 ± 1.2	3.6 ± 0.7	3.8 ± 1.2
lipid ratio	1 month	3.7 ± 0.5	4.0 ± 0.8	3.8 ± 1.0
(μmol/l /	3 months	4.2 ± 1.0	4.0 ± 0.9	3.6 ± 0.8
mmol/l)	6 months	3.9 ± 0.7	4.1 ± 0.5	3.3 ± 0.9
	12 months	3.7 ± 0.9	3.9 ± 1.1	3.2 ± 0.6 [d]
Ascorbic acid	Baseline	44 ± 24	36 ± 18	39 ± 20
(μmol/l)	1 month	47 ± 16	33 ± 17	44 ± 14
	3 months	37 ± 19	39 ± 26	52 ± 20 [e]
	6 months	40 ± 20	48 ± 20	39 ± 21
	12 months	50 ± 19	45 ± 22	38 ± 19
Uric acid	Baseline	404 ± 103	365 ± 118	316 ± 81
(μmol/l)	1 month	493 ± 154 [e]	439 ± 105 [e]	290 ± 97
	3 months	392 ± 75	382 ± 112	303 ± 98
	6 months	352 ± 55 [e]	352 ± 100	310 ± 55
	12 months	347 ± 94 [e]	339 ± 81	275 ± 74

Means were significantly different from the baseline values, within the groups: [a] $p < 0.0001$; [b] $p < 0.02$; [c] $p < 0.03$; [d] $p < 0.05$; [e] $p < 0.007$.
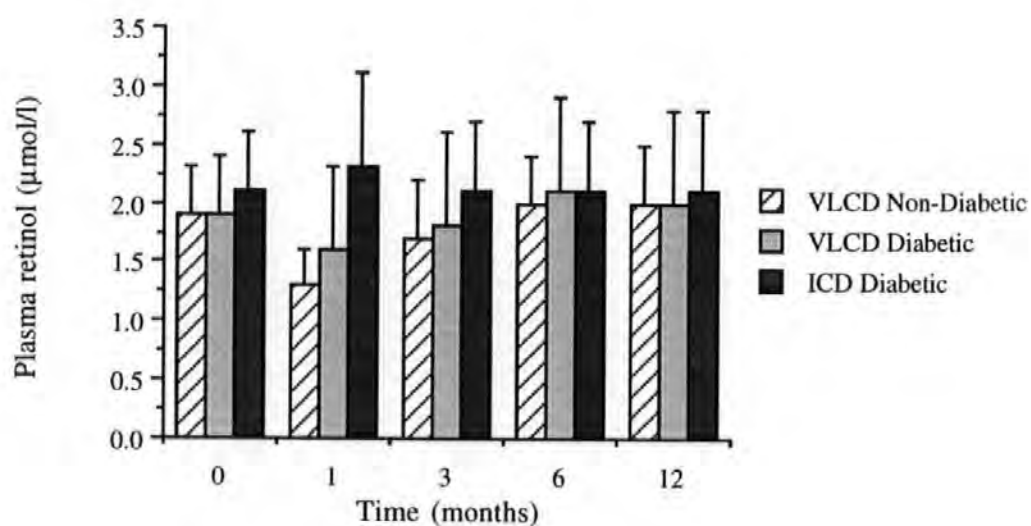
Figure 7.14 *Changes in plasma retinol concentrations.*
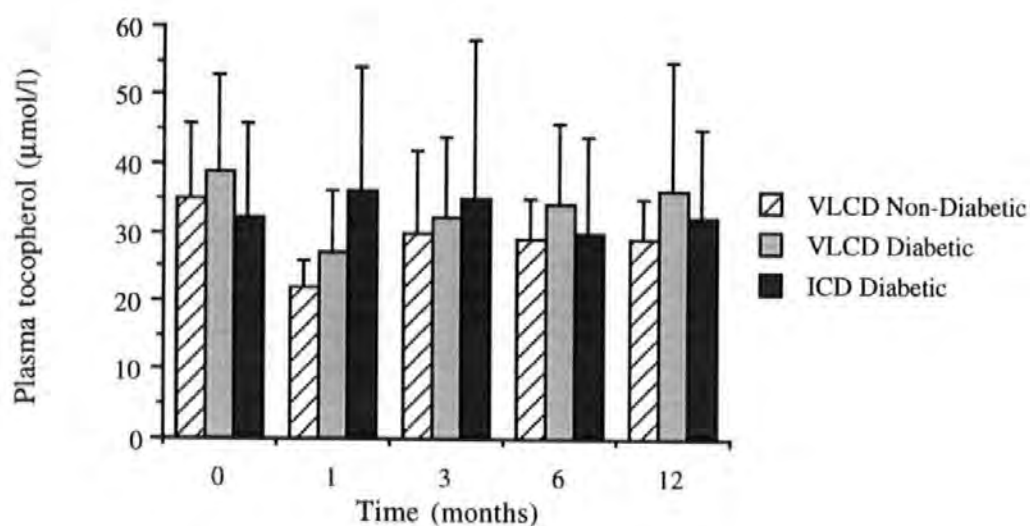
Figure 7.15 *Changes in plasma α-tocopherol concentrations.*

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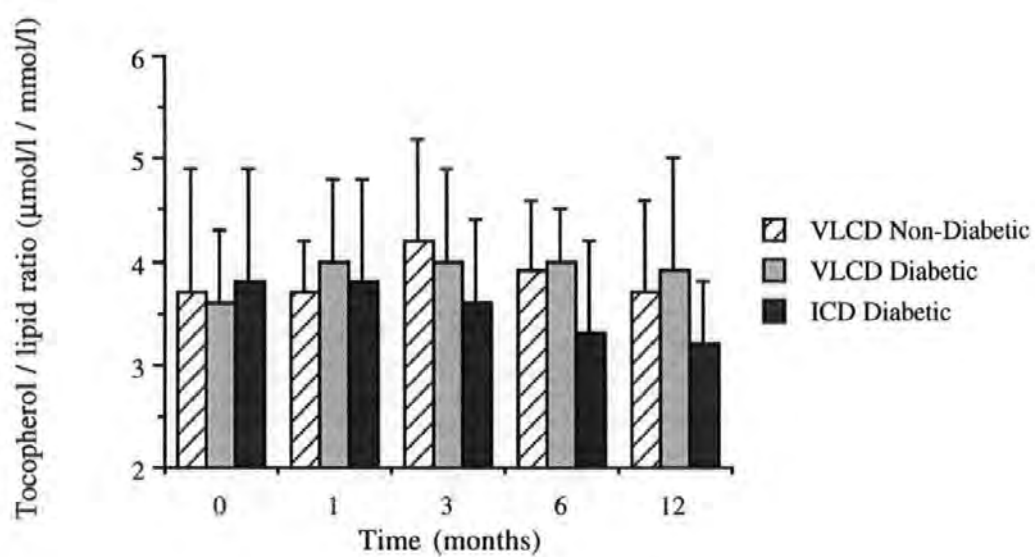
Figure 7.16 *Changes in α-tocopherol / triglyceride + cholesterol ratios.*

(ii) Water-soluble antioxidants

Plasma ascorbate concentrations were slightly higher in the non-diabetic group compared with the two diabetic groups at baseline (Figure 7.17). There were no significant changes in plasma ascorbate concentrations in the two VLCD groups during the 12 months of follow-up. However, it was interesting to record that plasma ascorbate concentrations were significantly higher in the non-diabetic subjects compared with the diabetic subjects on VLCD at 1 month ($p = 0.02$). The daily intakes were the same in each group at this time, i.e. 60 mg per day for women and 80 mg per day for men, whilst consuming the diet. In the ICD group, plasma ascorbate concentrations showed a gradual increase reaching significance at 3 months ($p = 0.03$). No correlations between plasma ascorbate concentrations and indices of glycaemic control (post-prandial plasma glucose and serum fructosamine) were found in the patients studied.

The changes in serum urate are shown in Figure 7.18. Urate concentrations remained stable in the ICD group during the 12 months of follow-up. There were significant increases in serum urate concentrations in the non-diabetic and diabetic subjects after one month of VLCD ($p < 0.007$). Thereafter, urate levels gradually returned to baseline values, although at 6 and 12 months urate levels were significantly lower than baseline in the non-diabetic group ($p < 0.007$). When the groups were compared, urate levels were found to be significantly higher in the two VLCD groups compared with the ICD group at 1 month ($p = 0.001$).
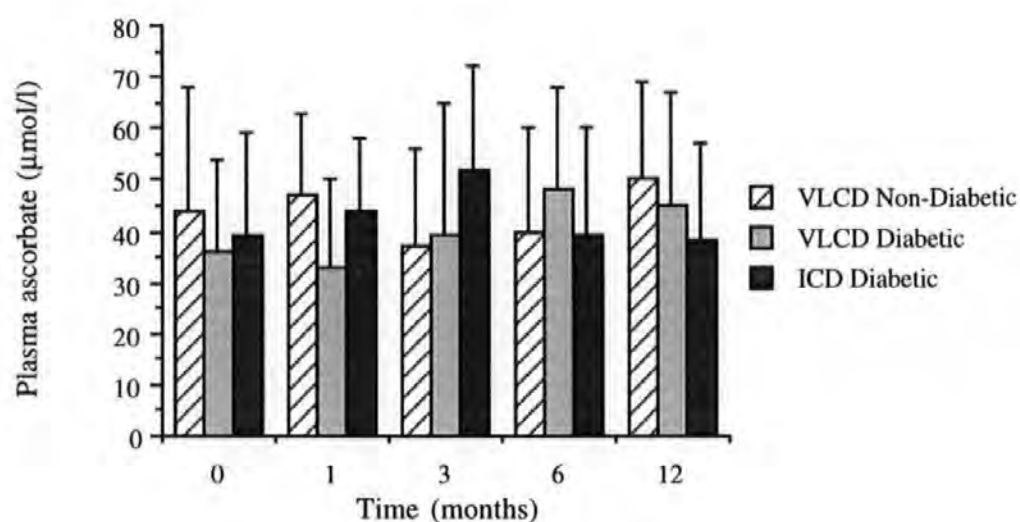
Figure 7.17 *Changes in plasma ascorbate concentrations.*
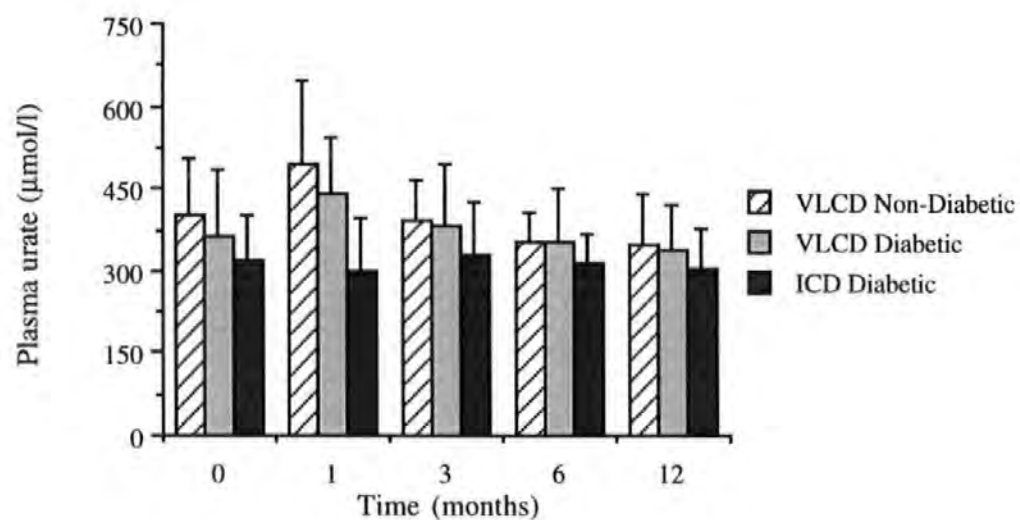
Figure 7.18 *Changes in plasma urate concentrations.*

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7.5.4 Plasma lipid peroxides

Figure 7.19 shows the changes in plasma MDA concentrations during the 12 months of follow-up. There were no significant differences between the groups at baseline, although plasma MDA concentrations were slightly higher in both of the diabetic groups compared with the non-diabetic control group. After 1 month of dieting, plasma MDA levels had decreased significantly in both groups on the VLCD ($p < 0.004$) and were also significantly lower than those of the ICD diabetic patients at this time ($p < 0.02$). The MDA concentrations remained lower than the baseline levels throughout the remainder of the follow-up period in both VLCD groups and were also significantly lower than the baseline levels after 6 months in the non-diabetic group ($p = 0.018$). After 12 months, plasma MDA levels were on average 0.1 μmol/l lower in the non-diabetic patients and 0.16 μmol/l lower in the diabetic patients on the VLCD, although the decreases were not statistically significant.

In contrast, a gradual increase in MDA levels was observed in the ICD diabetic patients and after 12 months plasma MDA levels were on average 0.14 μmol/l higher than the baseline values, although the increases were not statistically significant. Furthermore, MDA levels were also significantly higher in the ICD group than in the non-diabetic VLCD group at 6 months ($p = 0.018$) and after 12 months MDA levels were on average 0.4 μmol/l higher in the ICD group than the two VLCD groups (not statistically significant).

The changes in plasma MDA concentrations in relation to the changes in serum lipids (the sum of the triglycerides and cholesterol) are shown in Figure 7.20. At baseline, plasma MDA / lipid ratios were higher in the diabetic patients in the ICD group compared with the VLCD diabetic group ($p = 0.052$) and significantly higher than those of the non-diabetic group ($p = 0.02$). Interestingly, despite the large reduction in serum lipids after 1 month of the VLCD, the proportion of MDA to serum lipids increased in both VLCD groups and the increase was significant in the non-diabetic group ($p < 0.008$). Thereafter, plasma MDA / lipid ratios returned to the baseline levels. In the ICD group, the MDA / lipid ratios remained stable, but also remained higher than the VLCD groups.
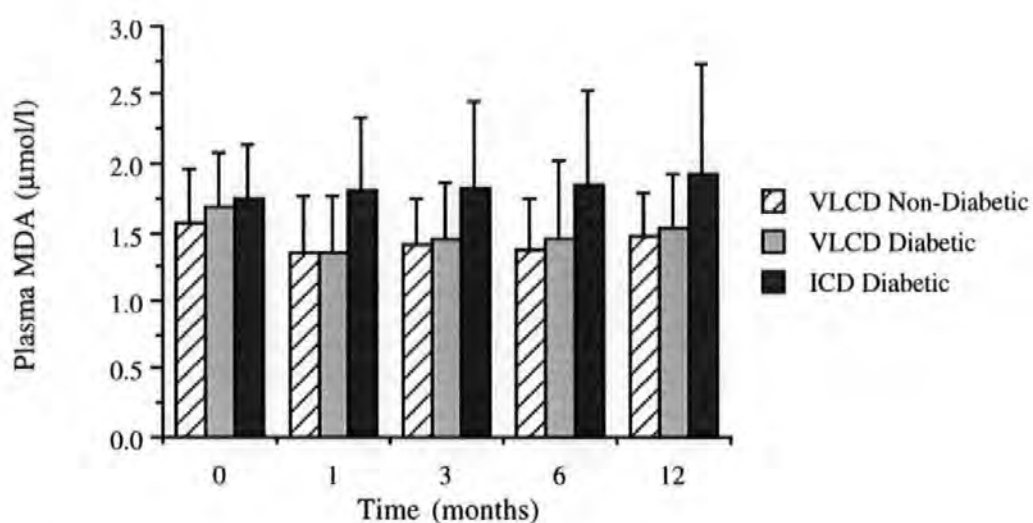
Figure 7.19 *Changes in plasma MDA concentrations.*
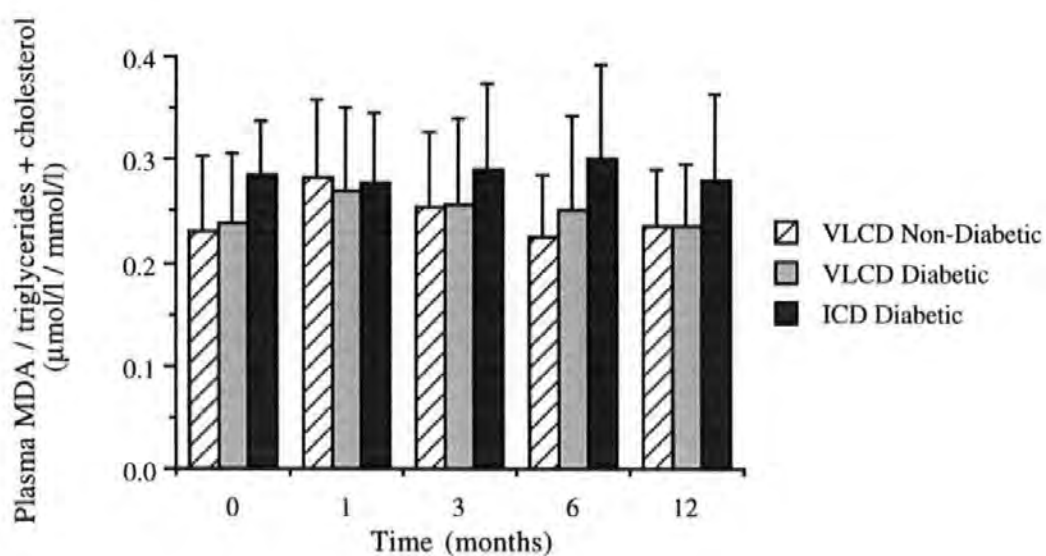
Figure 7.20 *Changes in MDA / triglyceride + cholesterol ratios.*

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The conjugated diene / linoleic acid ratio was measured in 11 patients in each VLCD group, but only in 4 patients in the ICD group. The changes in the conjugated diene / linoleic acid ratio are shown in Figure 7.22. At baseline, the conjugated diene ratios were higher in the diabetic groups compared with the non-diabetic group VLCD group, although the differences were not significant. After 1 month, the ratios decreased in both VLCD groups and at 3 months the decrease was significant in the diabetic VLCD group ($p < 0.05$). In comparison, the ratios remained stable in the ICD group and showed a slight worsening at 6 and 12 months.

At baseline, plasma MDA levels were significantly higher in the three patients groups compared with a healthy non-obese control group ($p < 0.001$). Furthermore, regardless of the initial improvements that were found, plasma MDA levels remained significantly higher than those of the healthy control group in all of the patient groups and at all of the follow-up times ($p < 0.015$). However, plasma MDA levels were returned to the reference range in the two newly diagnosed patients as a result of the dietary restriction of the VLCD.

The results indicated that MDA levels were increased in obese non-diabetic patients; thus, relationships between MDA levels and other parameters associated with obesity were investigated. No correlations between plasma MDA concentrations and weight or BMI were found, although weak positive correlations between MDA levels and waist / hip ratios were found at 6 and 12 months ($r = 0.34$ and 0.39, respectively, $p < 0.05$). Interestingly, no correlations were found between plasma MDA levels and age, therefore, no adjustments for age were necessary. Correlations between plasma MDA levels and glucose and fructosamine concentrations indicated weak positive associations at 1 month only ($r = 0.34$, $r = 0.38$, respectively, $p < 0.05$), when the largest decreases in plasma MDA and glucose occurred.
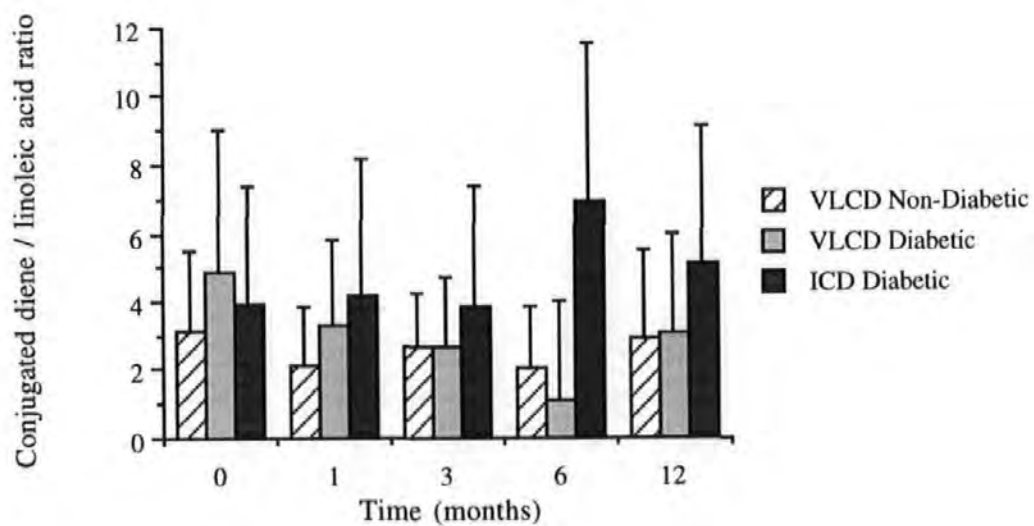
Figure 7.21 *Changes in the conjugated diene / linoleic acid ratios.*

7.6   Discussion

Weight loss is recommended for obese patients, especially patients with type II diabetes, in order to improve metabolic control, reduce cardiovascular risk factors and lessen the need for intensive therapies such as insulin. In particular, weight loss during the first year after diagnosis can lead to the reversal of type II diabetes and increase life expectancy (Pories *et al.* 1995; Lean *et al.* 1990). Modest weight losses in patients with established type II diabetes are equally beneficial for producing long-term metabolic improvements (Wing *et al.* 1987a; Rotella *et al.* 1994). However, the increasing prevalence of obesity, worldwide, together with the associated increase in type II diabetes and the morbidity and mortality which this condition incurs, has increased the urgency for the use of aggressive weight loss therapies, such as VLCDs.

In the 1970s and 80s, concern about the safety of VLCDs arose due to reports of sudden deaths on formulated diets, as a result of the poor protein and micronutrient content of some of these diets. Concern was also expressed about the losses in lean body mass whilst on VLCDs, which has resulted in the reformulation of these diets, so that today's VLCDs are regarded as safe when used correctly (DHSS 1987; Wadden *et al.* 1990; NTFPTO 1993). This study has examined the effects of a VLCD and ICD therapy on cardiovascular risk factors and indices of oxidative stress in obese type II diabetic and non-diabetic patients, in order to provide further information on the safety and efficacy of these weight loss strategies.

Weight loss

At the recruitment stage of the study, several patients expressed the desire to pursue the VLCD therapy, whilst others insisted on recruitment into the ICD groups. As a result, the patients were not randomized, but were offered the choice of the VLCD or the ICD therapy in order to maximize compliance. The treatments were available for the duration of the study. Consequently, the morale of the patients was high at the start of the interventions.

The first week of the VLCD was the most difficult stage in this dietary therapy, so that once over the initial adjustment to the diet, the majority of the patients experienced little difficulty in following the Lipotrim protocol. Thereafter, the rapid weight loss that was experienced and the feeling of 'well-being' whilst on the diet, together with the group support, encouraged the patients to persevere with the programme. Consequently, the groups remained motivated during the acute weight loss phase and compliance with the diet was good, as judged by the presence of urinary ketones. The knowledge that the group support would be available during the weight maintenance phase of the programme was also reassuring for the patients stopping the VLCD.

As expected, rapid weight loss was experienced by the patients on the VLCD. Both VLCD groups lost significantly more weight than the ICD group during the period of acute weight loss (0-6 months, $p = 0.00001$), but also after 12 months ($p < 0.02$). Interestingly, the non-diabetic patients lost significantly more weight than the diabetic patients on the VLCD at 3 months (a mean loss of 20.7 kg in the non-diabetic group at 3 months, compared with a mean loss of 15.2 kg in the diabetic group ($p = 0.04$)). The duration on the diet was slightly longer in the non-diabetic group, which might have been responsible for part of this difference, although it is possible that behavioural differences between the groups also existed towards the diet. Indeed, Wing *et al.* (1987b) and Amatruda *et al.* (1988) have reported that weight loss in type II diabetic patients was more difficult than that for non-diabetic patients.

Weight losses were slower in the ICD group, which was disheartening for the patients who did not reach certain target weights within the time scales that had been set. This resulted in the crossover of three patients from the ICD groups to the VLCD groups at an early stage in the study. These observations indicated that it would have been better to ignore target weights and to encourage the patients to concentrate their efforts on losing weight steadily, i.e. 0.25-0.5 kg per week. Indeed, recent guidelines for the management of obesity recommend modest weight loss and weight maintenance as the main objectives of weight loss strategies, rather than focusing on reaching target weights (SIGN 1996).

Despite the slow rate of weight loss, the ICD group did achieve a significant reduction in weight at 6 months (3.4 kg, $p < 0.02$), but this was not maintained at 12 months. At first sight, the reduction in weight in our diabetic group appears poor in comparison with other behavioural studies. For example, in one study conducted by Wing *et al.* (1991), type II diabetic patients assigned to a 20 week behavioural programme achieved weight losses of 10 kg. A second group of diabetic patients consumed a VLCD for 8 weeks as part of the 20 week behavioural programme and achieved weight losses of 18 kg, which was also slightly better than our diabetic group (15 kg). However, the weight loss achieved by our ICD diabetic group was similar to that reported by Blonk *et al.* (1994), i.e., 2.9 kg at 6 months, whose comprehensive weight reduction programme was similar to ours. Furthermore, the weight losses achieved by our ICD group at 6 (3.4 kg) and 12 months (2.3 kg) were greater than those reported in diabetic patients allocated to conventional dietetic programmes, indicating the benefit of the ICD intervention. In the study of Blonk *et al.* (1994), patients receiving conventional treatment comprising of clinic visits at 2 monthly intervals, achieved a weight loss of 1.2 kg after 6 months. Similar weight losses were reported by Manning *et al.* (1995) at 12 months (1.2 kg), in diabetic patients attending dietetic clinics at 6 weekly intervals. Thus, the modest weight losses observed in our ICD group, in comparison with the study of Wing *et al.* (1991) may be accounted for by treatment differences. These results indicated that more behavioural strategies would need to be incorporated into our ICD treatment in future studies, in order to produce weight losses of 10 kg within 6 months.

The diabetic patients in our study had a duration of diabetes greater than 5 years, with the exception of two newly diagnosed patients, and were patients in whom previous attempts at dieting had failed. Thus, the modest weight losses achieved, emphasized the difficulty for weight reduction in this group of patients — an observation made by Kelly West in 1973. Indeed, two patients in the ICD group gained weight during the first 6 months of the study and required insulin therapy. Interestingly, analysing the data without these patients indicated that the significant weight loss that was achieved at 6 months (4.2 kg) was maintained at 12 months (3.7 kg, $p < 0.01$) which was encouraging.

During the weight maintenance phase of the VLCD programme, 11 non-diabetic patients and 4 diabetic patients experienced gradual weight regain, which was greater than 5 kg. Out of these patients, 4 non-diabetic and 1 diabetic patient regained more than 10 kg by 12 months. Concern about the rapid regain in weight after stopping VLCDs has been raised in the past (Wing 1992; NTFPTO 1993). In this study, the importance of the weight maintenance phase of the VLCD therapy was emphasized as part of the Lipotrim programme. However, once the patients had stopped the VLCD and transferred to maintenance diets, the attendance rates declined. Consequently, this factor may have contributed to the failure of these patients to maintain their weight losses. It was interesting to note that the non-diabetic patients lost more weight than the diabetic patients on the VLCD, but also regained more weight than the diabetic group. Thus, despite more difficulties in losing weight, the diabetic patients appeared better at maintaining their weight loss than the non-diabetic patients.

After the 12 month follow-up, 14 non-diabetic patients and 5 diabetic patients, who experienced gradual weight regain after stopping the VLCD, attempted to restart the diet. Repeat courses of the VLCD were available to patients who required to lose more weight, although the patients were informed that second attempts at the VLCD were usually more difficult than the first. Out of the 19 patients who attempted to restart the VLCD, only 6 did so successfully and 9 patients had more than one failed attempt at restarting the diet. Thus, it became apparent that to restart the VLCD a second time was much more difficult than had been anticipated. Smith and Wing (1991) have also reported that weight losses were smaller in patients attempting repeat courses of VLCDs. This was attributed to behavioral factors which resulted in poor patient compliance and diminished adherence to the diets, rather than physiological factors. Hence, future studies incorporating VLCDs into weight loss programmes may need to consider single courses of VLCDs with greater emphasis on the maintenance phase and stricter follow-up of patients after stopping the VLCD. Indeed, the weight maintenance phase of all weight loss therapies remains the most challenging area in the treatment of obesity. Wing and Greeno (1994) recently proposed that treatment programmes incorporating VLCDs should be lengthened and subgroups of patients with eating disorders identified and provided with additional support. A more chronic approach

to the treatment of obesity was recommended, with continued patient contact as an important component of future strategies.

BMI

The rapid weight loss produced by the VLCD resulted in significant reductions in BMI in the two VLCD groups at all of the follow-up times (Table 7.2, page 171). The weight loss in the ICD group produced only a one unit reduction in BMI between 1 and 6 months, with only 14% of the patients achieving a BMI ≤ 30. However, this was only transient and by 12 months only 7% of the patients had maintained their BMI ≤ 30. Interestingly, the faster rate of weight loss in the non-diabetic patients compared with the diabetic patients on the VLCD resulted in a greater proportion of patients achieving a BMI ≤ 30 at the end of the acute weight loss phase (i.e., 67% of the non-diabetic patients compared with 47% of the diabetic patients at 6 months). However, the regain in weight in the non-diabetic subjects by 12 months resulted in only 33% of the patients maintaining their BMI at 30 or below. In contrast, the proportion of diabetic patients with a BMI ≤ 30 had increased to 60% by 12 months. This favourable result provided a further indication that the diabetic patients were better at maintaining their weight losses than the non-diabetic patients on the VLCD and that a larger proportion of patients were continuing to lose weight after the end of the acute weight loss phase. Thus, the VLCD therapy was beneficial in terms of reducing BMI, particularly in the diabetic patients by the end of the first year of the study.

Waist-hip ratios

The reduction in BMI was accompanied by significant reductions in waist circumferences at 3, 6 and 12 months in both VLCD groups (Table 7.2) and waist / hip ratios were also significantly lower in the diabetic group by 12 months ($p < 0.05$). In contrast, the weight loss produced by the ICD therapy was too small to make any difference to the waist measurements or to the waist / hip ratios.

A recent study has emphasized that waist / hip ratios ≥ 0.95 in men and ≥ 0.80 in women

identify individuals with a BMI ≥ 25 and hence at increased risk from cardiovascular disease (Han *et al.* 1995). Despite the significant reductions in waist measurements and waist / hip ratios, the waist / hip ratios were still high in men (with a mean of 0.95 in the non-diabetic men and 0.97 in the diabetic men at 12 months) and women (with a mean of 0.84 in the non-diabetic women and 0.88 in the diabetic women) in the VLCD groups. Thus, the high waist / hip ratios at 12 months reflected the high BMIs and indicated that further weight loss was necessary in both VLCD groups in addition to the ICD group.

Blood pressure

Large reductions in systolic and diastolic blood pressures were found in the non-diabetic patients on the VLCD at all follow-up times (Table 7.2, page 171). A maximum reduction in systolic blood pressure of 19 mm Hg was seen at 3 months and diastolic blood pressures were reduced by 10 mm Hg at 12 months. Thus, the health benefits in relation to the reduced risk of stroke and coronary heart disease were clear, if the reductions were to be maintained long-term. Remarkably, systolic blood pressures only showed a significant reduction of 10 mm Hg at 1 month in the diabetic patients on the VLCD and diastolic blood pressures remained stable throughout the period of observation, although the patients had stopped their anti-hypertensive therapies by the first month. Systolic blood pressures remained stable in the ICD group, but diastolic blood pressures were lowered significantly after 1 month and a reduction of 8 mm Hg was maintained after 12 months. Hence, despite the lower weight losses in the ICD group, the intensive conventional dietetic advice offered to the patients resulted in significantly greater reductions in diastolic blood pressures than the VLCD therapy after 6 and 12 months ($p < 0.02$). This reduction in diastolic blood pressure was possibly related to increased levels of exercise and reduced salt intake in the ICD group, although these were not measured and the patients remained on their anti-hypertensive medications.

Previous studies have shown than weight reduction with VLCD therapy reduces blood pressure (Wadden and Stunkard 1986; Amatruda *et al*. 1988). The reduction in blood

pressure has been reported to be mediated by the low salt intake of the VLCD, the loss of oedema fluid, reduced SNS activity and the weight loss itself. Interestingly, the change in weight correlated positively with the change in systolic blood pressure at 1 month ($p < 0.05$) and 3 months ($p < 0.01$) and with the change in diastolic blood pressure at 1 month ($p < 0.05$) in the non-diabetic subjects. A positive correlation between the change in weight and the change in diastolic blood pressure was also found in the ICD group at 1 month ($p < 0.05$), but no correlations were found in the diabetic patients on the VLCD. Thus, hypertension responded to the weight loss in the non-diabetic patients, but not as dramatically in the diabetic patients on the VLCD. These differences reflected different physiological processes producing hypertension in the two groups. Insulin resistance and hyperinsulinaemia probably played a greater role in hypertension in the diabetic patients and although insulin sensitivity improved — judged by the fact that the patients had remained off their anti-diabetic medication — this was not sufficient to reduce blood pressure in this group. Furthermore, despite the large reduction in weight that was achieved by the VLCD diabetic group during the first year, the patients were still overweight and borderline obese, hence further weight loss and exercise may have been necessary in order to produce a greater reduction in blood pressure in this group.

Glycaemic control

Post-prandial plasma glucose and fructosamine levels were significantly reduced in the diabetic patients on the VLCD at one month ($p < 0.001$); the levels were also significantly lower than those of the ICD group at this time ($p < 0.03$). The improvements in glycaemic control were lost by 6 months when all, but one, of the patients had entered the refeeding stage. Thus, the weight loss did not produce long-term benefits in relation to glycaemic control in the VLCD diabetic group. However, the patients remained off anti-diabetic medication at 6 and 12 months, which suggests that there were improvements in insulin sensitivity in this group and the patients were still overweight at 1 year. In comparison, the slower rate of weight loss in the ICD group produced no change in glycaemic control and patients were not able to discontinue their medication.

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The improvement in glycaemic control in the diabetic patients, after 1 and 3 months of the VLCD, occurred as a result of the caloric restriction, which would have decreased hepatic glucose output and increased insulin sensitivity (Henry *et al*. 1985; Henry *et al*. 1986a; Laakso *et al*. 1988). Interestingly, despite the large reduction in weight and weight maintenance at 6 months, this was not enough to prevent glycaemic control from deteriorating and returning to baseline levels at 6 and 12 months in the VLCD diabetic group. Wing *et al*. (1991) have reported that long-term improvements in glycaemic control were probably the result of restored ß-cell insulin-secretory capacity, which occurred during the period of caloric restriction. Long-term improvements in glycaemic control were therefore possible in patients who could increase their insulin secretion in response to the increased caloric intake after refeeding and could therefore overcome insulin resistance. However, as described by Blonk *et al*. (1994) some patients may not be able to maintain glycaemic control despite maintaining substantial weight losses, as a result of the diminishing capacity of the pancreas to secrete insulin. The patients in our study were still overweight and bordering on obese, thus further weight reduction was probably necessary for reducing plasma glucose levels further, although the long-term improvements in glycaemic control would ultimately be determined by the capacity of pancreas to secrete insulin.

As expected, the non-diabetic patients on the VLCD showed no change in glycaemic control, nevertheless, glycaemic control appeared strikingly stable in comparison with that of the diabetic groups (Figures 7.8 and 7.9, page 180). It was also interesting to note that despite one month of 'fasting' whilst on the VLCD, plasma glucose concentrations remained significantly greater in the diabetic patients compared with the non-diabetic patients, an indication of the permanent metabolic disturbance associated with established diabetes. However, two patients in our study, who undertook the VLCD treatment were newly diagnosed with type II diabetes. One patient lost a remarkable 54 kg of weight by 6 months, from a baseline weight of 144 kg (BMI of 56) and by 12 months the weight loss had reached 70 kg and the BMI 29. Furthermore, plasma glucose and fructosamine concentrations had returned to normal values by 6 months and the improvements were maintained at 12 months.

The second patient achieved a weight loss of 14 kg by 3 months, reducing their BMI from 32 to 26. Plasma glucose and fructosamine concentrations were normalized after 1 month. This patient maintained their weight loss at 12 months and glycaemic control remained within the normal range. The patients were taken off all anti-diabetic medication at the start of the VLCD and throughout the 12 months of follow-up. Thus, these results indicated that the rapid weight loss produced by the VLCD and the subsequent weight maintenance resulted in the reversal of type II diabetes in these patients.

Serum lipids

Serum triglyceride and cholesterol levels improved significantly after 1 month in both groups on the VLCD and were significantly lower than the ICD group at this time ($p < 0.04$), indicating the benefit of the caloric restriction. These results confirm the findings of previous studies using VLCDs (Henry *et al.* 1986b; Uusitupa *et al.* 1990b; Wing *et al.* 1991) The improvements in serum triglycerides were maintained in the non-diabetic patients at 12 months, but not in the diabetic patients on the VLCD. Although, triglyceride levels were on average 0.9 mmol/l lower at 12 months compared with the baseline levels in the diabetic group, indicating some clinical improvement as a result of the sustained weight loss. The improvements in serum cholesterol levels were transient in both VLCD groups, so that by 12 months cholesterol levels had returned to the baseline values. A similar observation was found by Wing *et al.* (1991).

Interestingly, a lowering in HDL cholesterol concentrations was observed after 1 month of the VLCD in both groups (Figure 7.12, page 183), although this was not significant, but in the ICD group a significant reduction in HDL concentrations was found after 6 months ($p = 0.01$). A similar observation was reported by Henry *et al.* (1986b). However, HDL cholesterol concentrations increased thereafter and were significantly higher than baseline levels after 6 months in the non-diabetic patients on the VLCD and after 12 months in the diabetic patients ($p < 0.01$). The HDL / cholesterol ratios also increased significantly after 1 month in the non-diabetic patients and after 6 months in the diabetic patients ($p < 0.01$).

These results confirm the view that changes in HDL cholesterol levels occur over a longer time and that long-term weight reduction was associated with an increase in HDL / total cholesterol ratios in patients with type II diabetes (Uusitupa *et al.* 1990b; Wing *et al.* 1991).

Serum cholesterol, triglyceride and HDL / total cholesterol ratios remained stable during the 12 months of follow-up in the ICD group. Interestingly, there was a positive correlation between the change in weight and the change in serum cholesterol at 1 month $(p < 0.001)$ and at 3 months $(p < 0.01)$ and also between the change in weight and the change in triglyceride levels at 1, 3 and 6 months $(p < 0.01)$ in the VLCD groups. Thus, the lack of a reduction in serum triglyceride and cholesterol levels or increase in HDL / cholesterol ratio was probably due to the insufficient weight loss in the ICD group.

Lipid-soluble antioxidants

Retinol

The baseline data revealed that plasma retinol levels were very similar in the three patient groups. Other authors have found raised plasma retinol levels in patients with type II diabetes compared with non-diabetic individuals (Krempf *et al.* 1991). However, the non-diabetic control-group patients in our study were obese individuals and this factor may account for these differences.

During the acute weight loss phase, plasma retinol concentrations showed significant decreases after 1 and 3 months on the VLCD in the non-diabetic patients $(p < 0.03)$ and after 1 month in the diabetic patients $(p < 0.02)$. Upon cessation of the VLCD, the retinol levels returned to the baseline values. In comparison, retinol concentrations remained stable in the ICD group at all times. However, the plasma values remained within the reference range at all times, indicating that there were no adverse effects of the VLCD or ICD therapies on retinol levels.

α-Tocopherol

Plasma α-tocopherol and α-tocopherol / lipid ratios were similar in all three groups at baseline, supporting an earlier study that showed that α-tocopherol levels were within the normal range in diabetic patients (Vandewoude *et al.* 1987). During the acute weight loss phase, plasma α-tocopherol concentrations decreased significantly after 1 month in both VLCD groups ($p < 0.007$) as a result of the rapid lowering in plasma triglyceride and cholesterol levels. Interestingly, α-tocopherol concentrations remained lower than the baseline values in the non-diabetic subjects after 6 and 12 months ($p < 0.03$). In comparison, the α-tocopherol concentrations remained stable in the ICD group throughout the study. Vitamin E levels were within the plasma reference ranges at all times, indicating that there were no adverse effects of either dietary intervention.

The α-tocopherol / lipid ratios showed a transient rise at 3 months in the VLCD non-diabetic group; a similar increase was observed in the VLCD diabetic patients, which was maintained after 12 months — although the increases did not reach statistical significance. Thus, the VLCD therapy produced a favourable result in terms of increasing the antioxidant protection of the serum lipids, especially in the diabetic group.

In the ICD group, the α-tocopherol / lipid ratios showed a gradual decline after 3 months of dietetic advice, which reached statistical significance after 12 months ($p < 0.05$). The ratios remained well above the values indicative of vitamin E deficiency (i.e., 1.59 μmol/l α-tocopherol / mmol/l lipid, Thurnham *et al.* 1986). Thus, the dietetic advice had a long-term lowering effect on plasma α-tocopherol concentrations. Although the reduction in α-tocopherol / lipid ratios did not place the patients at risk from vitamin E deficiency, the lowering of serum lipid antioxidant protection was unfavourable, especially in the diabetic group, considering that the serum lipid levels were not significantly reduced by the dietetic treatment. These results also indicated that reducing serum cholesterol and triglycerides concentrations was also important for increasing the serum lipid-soluble antioxidant levels, especially in diabetic patients.

Water-soluble antioxidants

Uric acid

Serum uric acid concentrations showed a significant increase after one month, in both groups on the VLCD ($p < 0.007$). This elevation was transient and after 6 and 12 months urate levels had declined and were significantly lower than the baseline values in the non-diabetic patients ($p < 0.007$). The increase at 1 month was possibly due to the excretion of excess purine bases associated with the reduction in adipose tissue and lean tissue mass, which accompanied the rapid weight loss. Elevated serum ketone levels may have also prevented the excretion of urate by the kidneys, thereby raising serum levels. Other studies using VLCDs have reported similar transient increases in urate (Kreitzman 1984; Wing *et al.* 1991).

The patients in our study were asymptomatic to this elevation in serum urate, although concern has been expressed for patients with a history of gout undertaking a period of VLCD (Kanders and Blackburn 1994). However, the Lipotrim protocol indicated that patients with a history of gout should be monitored closely and treated prophylactically with allopurinol if necessary.

Ascorbic acid

Numerous studies have reported reduced plasma levels of ascorbate in diabetic patients (Will and Byers 1996). Our baseline data revealed that plasma ascorbate levels were slightly lower in the two diabetic groups compared with the non-diabetic group, indicating a possible disturbance in ascorbate metabolism, but the differences were not significant. Detailed dietary assessments were not available on the three groups, thus, it was not possible to comment further on possible differences between the groups at this time.

Plasma ascorbate levels remained stable in both VLCD groups, during the acute weight loss phase and after 12 months. It was interesting to note that after 1 month on the VLCD, plasma ascorbate levels were found to be significantly lower in the diabetic group compared

with the non-diabetic group ($p = 0.02$). Both groups were consuming the VLCD at this time and daily intakes were therefore the same in each group. These results support the observations made by Sinclair *et al*. (1994) that plasma ascorbate concentrations are significantly lower in diabetic patients consuming similar diets to non-diabetic patients and, hence, add support to the hypothesis that the metabolism of vitamin C is altered in diabetes. Thus, formulated VLCDs may require greater amounts of vitamin C for diabetic patients.

Furthermore, plasma ascorbate concentrations showed a steady decline in one newly diagnosed diabetic patient, who remained on the VLCD for an extended period. The plasma ascorbate levels declined from the baseline value of 48 μmol/l to 34 μmol/l at 1 month, 13 μmol/l at 3 months, 28 μmol/l at 6 months, returning to 67 μmol/l at 12 months after refeeding. Thus, formulated VLCDs may also need to contain greater than the present 'recommended nutrient intakes' of vitamin C, especially for morbidly obese patients who may need to undergo extended periods of supervised VLCD therapy, which are greater than the usual 10-12 weeks and this requires further investigation.

In the ICD group, plasma ascorbate concentrations increased significantly by 3 months ($p = 0.03$), indicating a possible increase in the consumption of more fruit and vegetables by this group. Armstrong *et al*. (1996) also recently reported a significant increase in plasma ascorbate in newly diagnosed patients following 2 months of dietary advice. Hence, dietary advice was valuable for increasing plasma ascorbate levels.

Negative correlations between plasma ascorbate and plasma glucose concentrations have been found previously, with the suggestion plasma ascorbate levels may be reduced with increasing hyperglycaemia (Yue *et al*. 1990; Lysy and Zimmerman 1992). In this study, no correlations between ascorbate and plasma glucose or fructosamine levels were observed at any time. Thus, the reduction in plasma ascorbate observed at 1 month in the VLCD diabetic group did not appear to be associated with glycaemic control, suggesting that other mechanisms are responsible for the reduction in plasma ascorbate found in diabetes.

Lipid peroxides

Plasma MDA concentrations were significantly reduced after 1 month in both VLCD groups ($p < 0.004$), but no improvements in lipid peroxide levels were found in the ICD group. The conjugated diene / linoleic acid ratios showed similar changes supporting the MDA results. The effect of food restriction in rodents has been known for sometime to reduce lipid peroxide levels and increase life expectancy, supporting the hypothesis that free radicals are involved in the ageing process (Laganiere and Yu 1987; Harman 1993). The results from this study showed that caloric restriction can reduce lipid peroxide levels in human subjects. Indeed, the VLCD produced a favourable long-term lowering effect on plasma lipid peroxide levels and also normalized MDA levels in two newly diagnosed diabetic patients. The ICD therapy, however, was not sufficient to produce marked improvements in plasma lipid peroxides, indicating that greater dietary restriction was necessary.

The proportion of MDA to serum lipids increased during the acute weight loss phase and was greatest at 1 month in the non-diabetic patients and this coincided with the lowering in α-tocopherol levels. The MDA / lipid ratios also increased in the diabetic VLCD group at 1 month. However, it was encouraging to note that the increase in MDA / lipid ratios was not greater in the VLCD diabetic patients compared with the non-diabetic patients at this time. This favourable result suggested that despite having significantly lower plasma levels of ascorbate at this time, the diabetic patients were not at greater risk from developing oxidative stress than the non-diabetic patients. Measurement of the susceptibility of LDL to oxidation at 1 month might have provided a further interesting insight into the differences between the diabetic and non-diabetic patients at this time and perhaps provided an indication as to whether formulated VLCD should contain greater amounts of vitamin E. Thus, further studies are warranted in this area.

It was interesting to note that plasma MDA concentrations in the non-diabetic VLCD group were significantly higher than those of a non-obese healthy control group at baseline. Furthermore, in spite of the initial improvements in plasma MDA levels, as a result of the VLCD, plasma MDA concentrations in the non-diabetic patients remained significantly

higher than those of the control group at all times ($p < 0.015$). Serum lipid levels were not available on all of the control subjects, therefore it was not possible to compare the MDA / lipid ratios, which may have provided a greater insight into the differences between the groups. However, the results indicated that lipid peroxidation was increased in obesity. Thus, relationships between MDA and other parameters associated with obesity were explored. No correlations between plasma MDA levels, weight, age or BMI were found. Weak positive correlations between MDA and plasma glucose, fructosamine were only found at 1 month. Thus, plasma glucose concentrations did not appear to have a significant association with plasma MDA levels. However, there was a weak positive correlation between plasma MDA levels and waist / hip ratios at 6 and 12 months ($r = 0.38$, $p < 0.05$), providing an indication that abdominal obesity was associated with alterations in lipid peroxides levels. These results supported an earlier view that obesity is associated with abnormalities in lipid peroxidation (Van Gaal *et al.* 1995). The measurement of plasma MDA concentrations has been suggested as a tool in the identification of patients at risk from cardiovascular disease (Gallou *et al*. 1994a; Griesmacher *et al*. 1995). The results from this study indicated that caloric restriction was one pathway by which lipid peroxidation could be lowered in high risk groups.

7.7 Conclusion

The intensive conventional dietetic advice offered to type II diabetic patients in this study was effective in producing modest weight loss during the first 12 months of the treatment, but did not produce greater long-term losses than the VLCD therapy. The weight losses achieved by the ICD therapy were not great enough to have a beneficial effect on reducing BMI, waist / hip ratios or serum lipid profiles, although significant reductions in diastolic blood pressures were achieved.

The VLCD produced large and rapid weight losses in the non-diabetic and diabetic patients and this was accompanied by significant reductions in BMI, and waist / hip ratios, which were maintained at one year. Interestingly, the maintenance of the weight loss was greater in the diabetic patients than in the non-diabetic patients. The weight loss was strongly associated with the reduction in systolic and diastolic blood pressure in the non-diabetic patients, but not in the diabetic patients on the VLCD. However, despite the large reduction in weight, the patients in the diabetic group remained overweight indicating that further weight loss or exercise might have been effective in improving blood pressures in this group.

Rapid reductions in serum triglycerides and cholesterol concentrations were observed in both VLCD groups, although the benefits were greater in the non-diabetic patients, since the improvements in serum triglycerides were maintained at one year. Despite the rapid weight loss, the improvements in HDL cholesterol levels were gradual and the long-term reduction in weight in the VLCD groups was associated with significant increases in HDL / total cholesterol ratios, particularly in the diabetic group. Thus, the VLCD produced substantial improvements in cardiovascular risk factors in both groups which were maintained at 12 months. The improvements with regard to the reduction in blood pressure and serum lipids, were greater in the non-diabetic patients compared with the diabetic patients on the VLCD.

The VLCD produced a significantly greater improvement in short-term glycaemic control than the ICD therapy. Although substantial weight losses were achieved by the diabetic

patients on the VLCD, the maintenance of the weight loss at 12 months was not sufficient to prevent the deterioration of glycaemic control and the type II diabetes had become irreversible. Thus, neither therapy produced a long-term improvement in glycaemic control. However, plasma glucose concentrations were normalized and maintained at normal levels after 12 months in two newly diagnosed diabetic patients who lost weight rapidly on the VLCD. Thus, the rapid weight loss produced by the VLCD was successful in reversing type II diabetes in newly diagnosed patients. The weight loss produced by the ICD therapy was slow, indicating that future regimens may need to incorporate additional behavioural strategies in order to achieve a faster rate of weight loss for newly diagnosed patients.

The VLCD produced transient reductions in plasma retinol and α-tocopherol concentrations, but did not adversely affect the levels of these vitamins since they remained within the normal reference ranges. On the contrary, the proportion of α-tocopherol to serum lipids increased as a result of the rapid reduction in serum cholesterol and triglycerides produced by the VLCD. The improvements in α-tocopherol / lipid ratios were maintained at one year in the diabetic patients largely as a result of the long-term improvement in serum triglyceride and cholesterol levels. Thus, lowering serum triglyceride and cholesterol concentrations had a positive effect on increasing α-tocopherol / lipid ratios. Intensive conventional dietetic advice resulted in a long-term lowering of α-tocopherol / lipid ratios in the diabetic group, who exhibited no significant reductions in serum lipid levels. These results indicated the importance of reducing serum lipid levels in diabetic patients receiving dietetic advice, in order to increase lipid-soluble antioxidant protection.

Plasma ascorbate concentrations increased in the ICD group, indicating that dietary advice was beneficial for increasing plasma water-soluble antioxidant levels. Plasma ascorbate concentrations were significantly lower in diabetic patients than non-diabetic patients, whilst consuming the VLCD. These results indicated that formulated VLCDs may require higher concentrations of ascorbic acid for diabetic patients, particularly for patients who need to undergo extended periods of supervised VLCD therapy.

Plasma levels of lipid peroxides were significantly reduced in the VLCD groups, with long - term improvements. Thus, the caloric restriction of the VLCD was beneficial for decreasing free radical activity in obese non-diabetic and diabetic patients. Despite the modest weight loss in the ICD group, lipid peroxide levels remained stable, indicating that greater caloric restriction was necessary in order to reduce free radical activity. The diabetic patients were not at greater risk from oxidative stress than non-diabetic patients whilst on the VLCD. Thus, the VLCD and ICD therapies were safe for diabetic and non-diabetic patients and produced improvements in cardiovascular risk factors and indices of oxidative stress.

8. Final Discussion and Conclusions

Sato *et al.* (1979) first reported elevated levels of lipid peroxides in the serum from diabetic patients. Since then, numerous studies have confirmed their findings and also indicated that levels of lipid peroxides are greater in patients with complications (Appendix 3). Despite the increased knowledge about free radicals, it is still in debate whether free radicals are involved in the development of diabetic complications or arise merely as a consequence of the tissue damage.

The role of hyperglycaemia has been established as the leading cause in the development of complications in type I diabetes (DCCT 1993), although the pathways by which hyperglycaemia leads to complications are not fully understood. Numerous *in vitro* studies have unequivocally established that glucose is a source of free radicals, however, the role of such reactions in the development of oxidative stress *in vivo* is still under investigation.

The main problem in the study of free radicals *in vivo* has been the fact that free radicals are very short-lived and hence no direct methods exist for the direct measurement of free radicals in the clinical setting. The 'gold standard' technique of ESR is of limited clinical use and although other techniques such as nuclear magnetic resonance or GC-MS are available, these are limited to specialized laboratories and require a high level of technical expertise. Thus, the majority of clinical studies rely on following the 'footprints' of free radical reactions by monitoring products of free radical attack on lipids, proteins, carbohydrates or DNA. Indeed, this study has shown that the measurement of serum lipid peroxides continues to provide a useful indirect indication of free radical activity *in vivo*.

In conclusion, this study has attempted to increase the knowledge surrounding the role of hyperglycaemia during the development of oxidative stress *in vivo* and to investigate the safety of VLCD and ICD therapies on cardiovascular risk factors and indices of oxidative stress in obese patients during periods of weight loss.

In summary, the main conclusions are that:

- Large increases in serum lipids were accompanied by large increases in lipid peroxides in patients with severe hyperglycaemia or diabetic ketoacidosis. Patients were, therefore, at risk from oxidative stress during the metabolic disturbances which culminated in diabetic ketoacidosis.

- Plasma antioxidant defences were very resilient during the recovery period in patients requiring hospitalisation due to episodes of severe hyperglycaemia and ketoacidosis.

- Very low calorie diet and intensive conventional dietetic therapies were safe for the treatment of obesity in diabetic and non-diabetic patients. Diabetic patients were not at greater risk from developing oxidative stress than non-diabetic patients whilst on the VLCD.

- Caloric restriction lowers plasma levels of lipid peroxides in obese diabetic and non-diabetic patients.

- Plasma ascorbate levels were found to be significantly lower in diabetic patients compared with non-diabetic patients consuming the VLCD. Thus, the vitamin C content of formulated VLCDs may require adjustment for diabetic patients.

- Reducing serum lipid levels was beneficial for increasing α-tocopherol / lipid ratios.

Further work

There are many areas for further research in the field of free radical activity in diabetes. The role of glycoxidation and autoxidation reactions and their contribution to oxidative stress *in vivo* suggests further evaluation with specific markers of products of these reactions. The role of transition metal ions may be a crucial factor linking these reactions with those of lipid peroxidation. Future studies could therefore consider measuring metal ion status. Additionally, studies focussing on the use of antioxidant supplementation may also involve consideration of transition metal ion reducing therapies, since reducing iron levels may also have antioxidant benefits.

It should be remembered that type I and type II diabetes are distinct conditions and comparative studies may point to different adaptive physiological processes which combat oxidative stress in these patients. This may provide a further insight to the development of oxidative stress and provide target areas for treatment. Furthermore, monitoring the changes in patients with first degree relatives with diabetes and during the transition from non-diabetic through IGT to frank diabetes mellitus would provide a greater understanding of the development of oxidative stress.

The optimum antioxidant requirements of diabetic and non-diabetic patients may differ, especially with regard to vitamin C. As indicated in this study, formulated diets may not provide adequate amounts of vitamin C for diabetic patients, especially for newly diagnosed and morbidly obese patients who may require long periods of caloric restriction and this requires further investigation. Furthermore, studies measuring the resistance of LDL to oxidation may provide an insight as to whether antioxidant protection of LDL particles is altered in patients consuming VLCDs and thus provide an indication whether patients would benefit from higher amounts of vitamin E whilst on a VLCD. Studies comparing lipid lowering treatments with those providing antioxidant supplementation could also provide an interesting insight for increasing α-tocopherol / lipid ratios in plasma.

Appendices

Appendix 1   Tocopherol Nomenclature

The naturally occurring stereoisomers of α-tocopherol, formerly known as *d*-α-tocopherol should be designated RRR-α-tocopherol. The totally synthetic α-tocopherol, formerly known as *dl*-α-tocopherol, should be designated all-*rac*-α-tocopherol. Esters of tocopherols should be designated as tocopheryl esters (e.g., α-tocopheryl acetate) (Machlin 1991).

The IU, which is equivalent to 1 mg all-*rac*-α-tocopherol acetate is the accepted measure of biological activity (Machlin 1991). The weight / IU relationships for different tocopherols are shown below.

Weight / IU relationships of tocopherols.

Tocopherol (Other name)	Amount (mg)	IU
all-*rac*-α-tocopheryl acetate *(dl*-α-tocopheryl acetate)	1.0	1.00
all-*rac*-α-tocopherol (*dl*-α-tocopherol)	1.0	1.10
RRR-α-tocopheryl acetate (*d*-α-tocopheryl acetate)	1.0	1.36
RRR-α-tocopherol (*d*-α-tocopherol)	1.0	1.49
all-*rac*-α-tocopheryl acid succinate (*dl*-α-tocopheryl acid succinate)	1.0	0.89
RRR-α-tocopheryl acid succinate (*d*-α-tocopheryl acid succinate)	1.0	1.21

Appendix 2   Enolization

The process whereby the α-hydrogen atom, attached to the α-carbon (the carbon atom adjacent to the carbonyl group), moves to the carbonyl oxygen atom, is known as **enolization**   (Pine *et al.* 1981). Isomeric carbonyl and enol structures are tautomers, where the reversible interconversion of the isomers is associated with the actual movement of electrons, as well as one or more hydrogen atoms. The reaction reaches an equilibrium, indicated by the equilibrium constant for tautomerism (K_T)

$$
\underset{\text{Carbonyl}}{\overset{\displaystyle H}{\underset{\displaystyle |}{-\,C\,-\,C}}\!\!\diagdown^{\displaystyle O}}
\quad \overset{K_T}{\rightleftharpoons} \quad
\underset{\text{Enol}}{\diagdown C = C \diagdown^{\displaystyle OH}}
$$
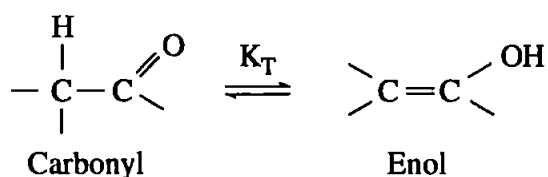
$$
K_T \;=\; \frac{[\text{enol}]}{[\text{carbonyl}]}
$$

The position of equilibrium depends upon the molecular structure. Normally the carbonyl form is favoured, but structural factors markedly affect K_T, e.g., 1,3 dicarbonyl compounds exist largely in the enol form at equilibrium. The enhanced stability of the enol form in 1,3-dicarbonyl compounds, compared with monocarbonyl compounds, is due to the formation of cyclic compounds.

Appendix 3   Studies of Free Radical Activity in Diabetic Patients

Reference	Subjects	Methods [a]	Results
Sato *et al.* 1979	110 Diabetic (Type unknown) 331 Control	TBARS + F	TBARS were significantly higher in the plasma of patients with complications compared with the control group and to those without complications ($p < 0.001$).
Nishigaki *et al.* 1981	31 Diabetic (Type unknown with complications) 32 Control	TBARS + F	TBARS were significantly higher in the diabetic group than in the control group ($p < 0.005$) and were found to reside mainly in the HDL fraction in serum.
Kaji *et al.* 1985	60 Type II (women) 71 Control (women)	TBARS + F Antioxidants	TBARS and GSH-Px, in plasma, were significantly higher in the diabetic group than in the control group ($p < 0.01$), the presence of complications was not specified. Erythrocyte GSH-Px, SOD and catalase levels were similar in the two groups.
Jennings *et al.* 1987a	62 Type I & II (36 no complications 26 microangiopathy) 36 Control	CD + S	CDs, in serum, were significantly higher in patients with microvascular disease compared with diabetics patients without complications and controls ($p < 0.001$). There was no difference between controls and patients without complications and no association between glycaemic control and CD levels.
Collier *et al.* 1988	34 Type I (with retinopathy) 35 Control	CD + HPLC	Plasma CDs were **significantly reduced** in diabetic patients with retinopathy compared with the controls ($p < 0.01$). No correlation between glycaemic control and CD levels was found.
Jain *et al.* 1989	21 Type I 17 Control	Erythrocyte TBARS + S TLC	TBARS were significantly higher in the membranes of erythrocytes from diabettic subjects (presence of complications was not specified) compared with the control subjects ($p < 0.05$). The degree of peroxidation correlated positively with the degree of glycaemic control.
Collier *et al.* 1990	22 Type II (without complications) 15 Control	Antioxidants	Erythrocyte SOD and plasma thiols were significantly lower in diabetic subjects compared with controls ($p < 0.01$). Erythrocyte thiols and plasma caeruloplasmin levels were similar in the two groups.

Continued...

[a] Methods used for the determination of lipid peroxides, as indirect indicators of free radical activity.
CD = Conjugated Diene; S = UV Spectrophotometry; F = Fluorimetry;
HPLC = High performance liquid chromatography; TLC = Thin layer chromatography.

Reference	Subjects	Methods	Results
Jennings *et al.* 1991	15 Type I (with retinopathy) 15 Type I (no complications) 15 Control	TBARS + S CD + S Antioxidants	Plasma TBARS were significantly higher in patients with retinopathy than controls ($p < 0.05$). CDs were raised, but not significantly, in both diabetic groups. Erythrocyte SOD and plasma thiols were significantly decreased in both diabetic groups ($p < 0.05$). No correlations were found between CDs, TBARS, SOD and glycaemic control.
Mooradian 1991	45 Type II elderly diabetic men (22 with and 23 without complications) 24 Control	CD + S	Serum CDs were significantly higher in patients with complications compared with the controls ($p < 0.01$). CDs in patients without complications were raised, but not significantly above those of the control group. Significant positive correlations were found between CDs and triglyceride and glucose levels.
Noberasco *et al.* 1991	67 Diabetic (20 Type I 47 Type II, free from acute illness) 40 Control	TBARS	Plasma TBARS were almost 2-fold higher in diabetic subjects than in the controls ($p < 0.01$). Subjects with poor glycaemic control had significantly higher TBARS than those with good glycaemic control ($p < 0.02$). A significant positive correlation was found between TBARS and fasting blood glucose.
Velázquez *et al.* 1991	18 Type II (with macrovascular disease) 20 Non-diabetic (with macro-vascular disease) 28 Control	TBARS + F	Both diabetic and non-diabetic subjects with macrovascular disease had significantly higher serum TBARS than the healthy controls ($p < 0.05$). Significant positive correlations were found between TBARS and triglycerides, cholesterol and glycaemic control, but no independent association between these variables and TBARS was found upon multiple regression analysis.
Armstrong *et al.* 1992	166 Type II (with complications) 51 Control	TBARS + F	Serum TBARS were significantly higher in diabetic than non-diabetic subjects ($p < 0.001$). TBAR levels corresponded to the prevalence rates of retinopathy and also showed a significant positive correlation with serum triglycerides and indices of glycaemic control. TBARS were lowest in patients treated by diet alone.

Continued...

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Reference	Subjects	Methods	Results
Collier et al. 1992	12 Type II (with microalbuminuria) 12 Type II (without microalbuminuria) 12 Control	CD + HPLC TBARS + HPLC	Plasma TBARS and CDs were higher in both diabetic groups than in the control group ($p < 0.05$). TBARS were also higher in the microalbuminuric group than in the normoalbuminuric group ($p < 0.05$). CDs and TBARS showed no correlations with glycaemic control.
Asayama et al. 1993	30 Type I Diabetic children 23 Control	AOA [b] Antioxidants	Serum AOA was lower in the diabetic group than in the controls ($p < 0.001$). Serum albumin and transferrin were also lower in the diabetic group ($p < 0.001$); caeruloplasmin and ferritin levels were unaltered.
	27 Type I Diabetic children 23 Control	TRAP [c] Antioxidants	Serum ascorbate and tocopherol levels were higher in the diabetic group than in the controls ($p < 0.05$), but the TRAP was lower in the diabetic group ($p < 0.001$).
Gallou et al. 1993	57 Type I 60 Type II 53 Control	TBARS + F	Plasma TBARS were higher in both diabetic groups than in the control group ($p < 0.001$), but no difference between Type I & II patients was found. Patients with complications had higher TBARS than those without complications $p<0.05$.
MacRury et al. 1993	13 Type II (with vascular disease) 12 Non-diabetic (vascular disease) 12 Control	TBARS + HPLC CD + HPLC Antioxidants	CD ratio, TBARS and caeruloplasmin were significantly increased in subjects with vascular disease, but no difference between diabetic and non-diabetics with vascular disease was found. Erythrocyte SOD levels were lower in both groups with vascular disease.
Chittar et al. 1994	117 Type II 34 Type I 30 Control	TBARS + F	Plasma TBARS were significantly higher in Type I and II diabetic patients than in the control subjects. Patients with complications had higher TBARS than those without complications.
Gallou et al. 1994a	204 Type II (with macrovascular disease) 107 Control	TBARS + F	Plasma TBARS were significantly higher in diabetic subjects (with or without vascular disease) compared with controls ($p <0.0001$). Patients with macrovascular disease also had significantly higher TBARS than those without vascular disease ($p < 0.0001$). TBARS correlated positively with total cholesterol.

Continued...

[b] Preventative antioxidant activity (AOA) was assessed by the ability of serum to inhibit lipid peroxidation of brain homogenates (expressed as the percentage inhibition of the production-rate of TBARS).

[c] The total peroxyl-radical trapping antioxidant parameter (TRAP) in serum.

Reference	Subjects	Methods	Results
Belch *et al.* 1995	19 Type I (with microangiopathy) 19 Non-diabetic (with vascular disease) 19 Control	TBARS + S Thiols	Plasma TBARS were higher in diabetic ($p < 0.01$) and non-diabetic subjects with vascular disease ($p < 0.05$) than controls. TBARS were also higher in the diabetic group than in the non-diabetic group with vascular disease ($p < 0.05$). Plasma thiols were lower in both patient groups compared with the controls ($p < 0.05$).
Gopaul *et al.* 1995	39 Type II 15 Control	GC-MS [d] Isoprostanes	Plasma levels of F_2-isoprostanes were higher in the diabetic patients compared with the control group ($p < 0.0001$). However, the presence of complications was not specified. The F_2-isoprostanes did not correlate with fasting plasma glucose or glycosylated haemoglobin, nor with triglycerides or cholesterol.
Griesmacher *et al.* 1995	77 Type I 81 Type II 62 Control	TBARS + F	Serum TBARS were significantly higher in diabetic patients (with and without vascular disease), compared with the controls ($p < 0.001$). Patients with Type II diabetes also had higher TBARS than patients with Type I diabetes ($p < 0.001$). TBARS were also higher in patients with poor glycaemic control, but no correlation between TBARS and glycaemic control was found.
Nacitarhan *et al.* 1995	78 Type II (with & without hyperlipidaemia) 38 Non-diabetic (with (hyperlipidaemia) 28 Control	TBARS + F	Serum and urinary TBARS were higher in diabetics (with and without hyperlipidaemia) than controls ($p <0.05$). Hyperlipidaemic diabetic patients had higher serum TBARS than normolipidaemic diabetic patients ($p < 0.02$). TBARS were also higher in hyperlipidaemic non-diabetics than controls ($p < 0.01$). No difference was found between the diabetic patients regarding complications. TBARS correlated significantly with serum glucose and cholesterol in the hyerlipidaemic diabetic group only.
Nourooz-Zadeh *et al.* 1995	22 Type II Control (number unknown)	ROOH [e] TBARS + S	Elevated plasma ROOHs were found in the diabetic subjects compared with the control group ($p <0.0005$), but TBARS were similar in both groups. Higher ROOHs were found in patients with, than those without, complications. No correlation between ROOHs, glycaemic control triglyceride or cholesterol was found.

Continued...

[d] GC-MS = Gas chromatography - mass spectrometry of prostaglandin F_2-like compounds (isoprostanes) - specific *in vivo* markers of non-enzymatic peroxidation of arachidonic acid.

[e] ROOHs were measured by the oxidation of ferrous to ferric ions using xylenol orange as an indicator.

Reference	Subjects	Methods	Results
Dandona *et al.* 1996	12 Type I 12 Type II 10 Control	8-OHdG *f*	8-OHdG levels in mononuclear cell DNA were higher in Type I and Type II diabetic patients than in control subjects ($p < 0.001$). However, this might have been as a result of increased generation of ROS by these cells.
Sundaram *et al.* 1996	467 Type II (with and without complications) 180 Control	TBARS + F Antioxidants	TBARS were more than 2 fold higher in the plasma and erythrocytes of diabetic subjects (regardless of complications) compared with controls ($p < 0.001$), but TBARS were also higher in those with complications than those without. Plasma vitamins C, E and GSH, and erythrocyte SOD and catalase, were significantly lower in diabetic subjects (regardless of complications) than controls, but erythrocyte GSH-Px levels were significantly higher in the diabetic group.
Ceriello *et al.* 1997	40 Type II (without complications) 40 Controls	TRAP TBARS + F Antioxidants	Plasma TRAP was lower in the diabetic group ($p < 0.001$) and lowest in those patients with poor glycaemic control, indicating that plasma from patients with Type II diabetes is more suscesptible to lipid peroxidation than plasma from healthy subjects. TBARS were higher in the diabetic group, but the difference disappeared after correction for serum lipids.
Maxwell *et al.* 1997	49 Type I 69 Type II	TRAP Antioxidants	Patients with Type I diabetes had significantly lower serum TRAP than patients with Type II diabetes ($p < 0.001$) —this was largely attributed to the lower levels of uric acid in the Type I diabetic group. A strong negative correlation between TRAP and glycosylated haemoglobin and urate levels was found only in patients with Type II diabetes, suggesting that poor glycaemic control is associated with reduced antioxidant activity in Type II diabetes.

f 8-Hydroxydeoxy guanosine (8-OHdG) was measured by HPLC as an indicator of oxidative damage to DNA.

Appendix 4   The Composition of Lipotrim

	Per serving- Women 38.3 g / sachet	Per serving- Men 76.6 g / sachet	Per 50 g Flapjack
kcal	135.0	270.0	200.0
protein -g	14.0	28.0	14.0
fat -g	2.7	5.4	11.0
carbohydrate -g	14.7	29.4	10.0
Vitamin A -mg	0.3	0.6	0.3
Vitamin B1 -mg	0.5	1.0	0.5
Vitamin B2-mg	0.6	1.2	0.6
Vitamin B6 -mg	0.7	1.4	0.7
Vitamin B12 -μg	1.0	2.0	1.0
Vitamin C -mg	20.0	40.0	20.0
Vitamin D3 -μg	3.0	6.0	3.0
Vitamin E -mg	3.3	6.6	3.3
Vitamin K -μg	47.0	94.0	47.0
Biotin -μg	67.0	134.0	67.0
Folic acid -μg	133.0	266.0	133.0
Niacin -mg	6.3	12.6	6.3
Pantothenic acid-mg	2.3	4.6	2.3
Calcium -mg	266.7	533.4	266.7
Chloride -g	0.5	1.0	0.5
Chromium- μg	42.0	84.0	42.0
Copper -mg	1.0	2.0	1.0
Iodine -μg	50.0	100.0	50.0
Iron -mg	7.0	14.0	7.0
Magnesium -mg	133.3	266.6	133.3
Manganese -mg	1.3	2.6	1.3
Molybdenum -μg	83.0	166.0	83.0
Phosphorous -mg	266.7	533.4	266.7
Potassium -g	1.17	2.34	1.17
Selenium -μg	42.0	84.0	42.0
Sodium -g	0.5	1.0	0.5
Zinc -mg	5.0	10.0	5.0

The main ingredients of the Lipotrim products were:

Sachets: skimmed milk powder, defatted soya flour, soya protein isolate, lecithin, tri-sodium citrate, dried glucose syrup, thickeners — carrageenan and xanthan gum, vegetable fat, monocalcium phosphate, potassium chloride, magnesium oxide, compound vitamins and minerals mixture, artificial sweetener — aspartamine, milk protein, colourings, flavourings and acidity regulator — potassium phosphate.

Flapjacks: peanuts, polydextrose, sodium caseinate, vegetable oil, textured soya protein, oats, fructose, soya bean flour, isolated soya protein, honey, cellulose, emulsifier — soya lecithin, monocalcium phosphate, dipotassium phosphate, flavouring, magnesium oxide, compound vitamins and minerals mixture, sea salt and sorbic acid.

	Lipotrim For Women		**Lipotrim For Men**	
	Per 3 servings	DRV* (RNI)	Per 2 - 3 servings	DRV (RNI)
kcal	405.0 - 470.0	**	540.0 - 670.0	**
protein -g	42.0 - 42.0	**	56.0 - 56.0	**
fat -g	8.1 - 19.4		10.4 - 27.4	
carbohydrate -g	39.4 - 44.1		39.4 - 58.8	
Vitamin A -mg	0.9	0.6	1.2	0.7
Vitamin B1 -mg	1.5	0.8	2.0	1.0
Vitamin B2 -mg	1.8	1.1	2.4	1.3
Vitamin B6 -mg	2.1	10.0	2.8	1.4
Vitamin B12 -μg	3.0	1.5	4.0	1.5
Vitamin C -mg	60.0	40.0	80.0	40.0
Vitamin D3 -μg	9.0	10.0	12.0	10.0
Vitamin E -mg	10.0	1.2	13.2	>4.0
Vitamin K -μg	141.0	1.0/ kg	188.0	1.0/ kg
Biotin -μg	201.0	10-200	268.0	10-200
Folic acid -μg	400.0	200.0	532.0	200.0
Niacin -mg	19.0	12-13	25.2	16-18
Pantothenic acid-mg	7.0	3-7	9.2	3-7
Calcium -mg	800.1	700.0	1066.8	700.0
Chloride -g	1.5	2.5	2.0	2.5
Chromium -μg	126.0	>25.0	168.0	>25.0
Copper -mg	3.0	1.2	4.0	1.2
Iodine -μg	150.0	140.0	200.0	1.0
Iron -mg	21.0	8.7	28.0	8.7
Magnesium -mg	400.0	270.0	533.2	300.0
Manganese -mg	4.0	>1.4	5.2	>1.4
Molybdenum -μg	249.0	50-400	332.0	50-400
Phosphorous -mg	800.1	550.0	1066.8	550.0
Potassium -g	3.5	3.5	4.7	3.5
Selenium -μg	126.0	60.0	168.0	75.0
Sodium -g	1.5	1.6	2.0	1.6
Zinc -mg	15.0	7.0	20.0	9.5

* In 1991, the Department of Health published new 'dietary reference values' (DRVs), replacing the old 'recommended dietary allowances' (RDAs) of 1979. The 'reference nutrient intakes' (RNIs) — the amount of a nutrient that will be sufficient for almost every individual, even those with high needs — are broadly quivalent to the old RDAs.

** Department of Health and Social Security recommendations (1987).

The Lipotrim product was intended for use as a total food replacement.

For women, the daily intake was in the form of three womens' sachets of Lipotrim (405 kcal/day) or two sachets and one flapjack (470 kcal/day).
For men, two mens' sachets of Lipotrim were taken daily (540 kcal/day), or if three meals were desired then one sachet and two flapjacks (670 kcal/day).

The sachets were mixed with approximately 250 ml of water and patients were advised to drink 2-4 l of water throughout the day. Tea and coffee with no added milk or sugar were permitted, but other beverages, e.g., milk or alcohol were not permitted whilst on the diet as these would prevent the development of the mild ketosis and cause hunger.

Refeeding Strategy
The main emphasis of the refeeding strategy was to reintroduce carbohydrates gradually into the diet over a period of days in order to prevent carbohydrate loading and return the glycogen levels to normal.

Patients were recommended to consume high protein, low fat and no carbohydrate on the first day of refeeding. Followed by high protein, modest carbohydrate and low fat on the second and third days. On the fourth day, modest amounts of complex carbohydrates could be introduced into the diet together with high protein and low fat.

Patients were given a list of the types of foods to consume and those to avoid.

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