ELECTRON SPIN RESONANCE AND EXERCISE-INDUCED OXIDATIVE STRESS: AN ANTIOXIDANT INTERVENTION STUDY

by

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A Doctoral Thesis

Submitted in canditure for the degree of
Doctor of Philosophy
of the University of Wales

February 1998

SUMMARY

Electron spin resonance (ESR) evidence concerning free radical production by exercise has been confined to animals. This thesis describes a methodology to measure free radicals using ESR in the venous circulation of healthy humans. It also describes three ESR studies of human blood to test the hypothesis that tissue damage associated with strenuous aerobic exercise is free radical-mediated due to the promotion of electron leakage from the mitochondrial electron transport chain via increased whole body oxygen flux, a mechanism known to result in free radical formation.

Study 1 demonstrated a significant increase in the concentration of the α-phenylbutyl-tert-nitrone (PBN) adduct (0.04 ± 0.01 vs 0.18 ± 0.04 arbitrary units, p=0.003) and ascorbyl radical (0.02 ± 0.001 vs 0.03 ± 0.002 arbitrary units, p=0.04) post-exercise. A threshold of increase of the PBN adduct appears to exist corresponding to approximately 70% VO_{2max} which may prove to be clinically important. Duplicate blood samples were analysed for lipid peroxidation including malondialdehyde (MDA) and lipid hydroperoxides (LH). MDA (0.70 ± 0.05 to 0.80 ± 0.04 $\mu mol.L^{-1}$, p=0.0125) and LH (1.15 ± 0.07 to 1.63 ± 0.29 $\mu mol.L^{-1}$, p=0.006) significantly increased post-exercise. Study 1 also demonstrated that exhaustive aerobic exercise results in significant increases in plasma endotoxin concentration (0.16 ± 0.03 vs 0.24 ± 0.06 $Eu.ml^{-1}$, p=0.001) pre vs post-exercise. This may be a free radical mediated phenomenon also of clinical significance.

Study 2 demonstrated that strenuous anaerobic exercise does not lead to increases in the concentration of the PBN adduct or the ascorbyl radical. Furthermore plasma LH did not change in this study although MDA increased significantly (0.74 ± 0.08 vs 1.73 ± 0.65 $\mu mol.L^{-1}$, p=0.01) pre vs post-exercise. This may reflect inadequacies in clearance of MDA.

Study 3 demonstrated that ascorbic acid supplementation results in an attenuation of the ESR signal both pre and post-exercise. Supplementation with ascorbic acid resulted in attenuation of exercise-induced lipid peroxidation and enhancement of blood antioxidant status. This research also demonstrates for the first time a complete abolition of endotoxin from the plasma of subjects who have undergone maximal aerobic exercise.

Additionally *in vitro* studies were performed in order to attempt to identify the origin of the radical species. These results suggest that the ESR signal of the PBN adduct is an oxygen-centred radical possibly derived from peroxidation of membrane PUFA. This work supports the hypothesis that strenuous aerobic exercise leads to increased free radical production probably via enhancement of mitochondrial electron transport chain leakage due to increased whole body oxygen uptake which is detectable using ESR spectroscopy.

ACKNOWLEDGMENTS

There are many people to whom I am deeply indebted for their advice and support in the execution of this research and the preparation of this thesis. This acknowledgement seeks to provide sincerest thanks to these people.

Primary among these people are Dr. Eleri Jones my Director of Studies, for giving me a chance in the first place and supporting me throughout, and Dr. Chris Rowlands my supervisor, also for unfailing support and encouragement, and guidance and scientific advice on ESR of the highest order.

To Professor Bruce Davies, for stimulating scientific discussions and much needed moral support.

To Dr. John Peters, Consultant Physician at the University Hospital of Wales Cardiff, for access to facilities at UHW and academic advice of the highest order.

I would like to acknowledge the excellent technical assistance of Dr. Ian Young of the Queens University, Belfast and Dr. Simon Jackson of the University of Wales College of Medicine.

To Professor RHT Edwards, Director of NHS Research and Development Wales, for providing a grant for a pilot study.

To my fellow postgraduates at the ESR laboratory, UWCC, for the good times especially Emyr Griffiths, Cormac Simms, and Lee Stokes.

This work would not have been completed without the help of the subjects who gave of their time (and their blood) freely and without complaint. I am truly grateful to all the subjects.

Finally, to my wife Claire, for patience and believing in me - thank you. Also for enduring many late nights while I was in the laboratory till the early hours, I'm sorry for all the burnt dinners !!

To you all - thank you.

I dedicate this thesis to my parents and my wife.

Thank you for all that you have done for me.

GLOSSARY OF TERMS.

The following are a series of working definitions referred to in this thesis:

Electron Spin Resonance (ESR) Spectroscopy	Analytical technique for the direct detection of free radical species.
Reactive Oxygen Species (ROS)	Unstable species derived from molecular oxygen, thought to be cytotoxic *in vivo*. (Includes free radicals and related substances).
Free radical	Chemical species containing an unpaired electron, the presence of which makes the species concerned highly reactive.
Antioxidant	Chemical substance that prevents oxidative damage caused by ROS *in vivo* eg. vitamin C.
Vitamin C	Generic name for substances exhibiting properties of ascorbic acid.
Lipid peroxidation	Process whereby unsaturated fatty acid molecules of membranes undergo attack and degradation by ROS.
Endotoxin	Lipopolysaccharide derived from cell wall of gram -ve bacteria. Possibly related to fever.

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"THOUGH IT COST YOU ALL YOU HAVE, GET UNDERSTANDING....."

Proverbs ch. 4: v. 7

Chapter One

INTRODUCTION

"For physical exercise profiteth little....."

1 Timothy ch. 4: v. 8

1.0 INTRODUCTION.

The health benefits of regular physical exercise may be largely beyond dispute (Shephard et al 1994). However, increased oxygen consumption during exercise may result in elevated production of free radicals leading to disturbances in homeostasis which are disruptive to normal cellular function.

Increased production of free radicals caused by exercise has been reported by several groups, including: Alessio and Goldfarb (1988); Kanter et al (1988); Sen et al (1994), using a range of techniques which are discussed later in this thesis (section 2.2, 2.3). The majority of these techniques focus on indirect assay of free radical damage such as malondialdehyde (MDA). Paramagnetic species such as free radicals are, by definition, highly reactive and thus their direct measurement in biological samples is particularly difficult. Electron spin resonance (ESR) spectroscopy has the ability to detect unpaired electron concentrations of a low level in biological and biochemical systems without destroying or modifying the substance in question (Ingram 1969), it is arguably the most sensitive and direct method of measuring free radicals.

Whilst there appears to be much conjecture in the literature that increased production of free radicals leads to exercise-induced tissue damage as determined by indirect measurements such as MDA, there has to date been no published ESR evidence describing the production of free radicals by exercise in the human venous circulation. Similarly there are no studies comparing aerobic exercise involving maximum oxygen uptake with exercise that is primarily anaerobic in relation to free radical production. Aerobic exercise causes increased oxygen flux possibly resulting in enhanced leakage of electrons from the mitochondrial electron transport chain which would, it is hypothesised, ultimately lead to an increase in the post-exercise ESR signal. In contrast

anaerobic exercise is not dependent on large increases in whole body oxygen uptake and thus should not lead to a post-exercise increase in the ESR signal intensity (amplitude). It is further hypothesised that any observed increase in the ESR signal intensity following exercise is thus free radical-mediated. Application of ESR to the measurement of free radicals in blood following aerobic and anaerobic exercise challenges should therefore provide valuable insight into the role of free radicals in exercise-induced tissue damage.

During exercise blood is redistributed from internal organs such as the gastro-intestinal tract to the working muscles. The gut may thus be in a state of relative ischaemia. One possible consequence of this may be increased free radical production on reperfusion leading to damage of the intestinal mucosa. This may allow translocation of bacterial lipopolysaccharide (endotoxin) into the systemic circulation. There appears to be no published studies examining the production of bacterial endotoxin during short-term exercise eliciting maximal oxygen uptake. It is thus hypothesised that this exercise-induced systemic endotoxaemia is also free radical-mediated.

One means of testing this hypothesis is that antioxidant supplementation should prevent the post-exercise increase in the ESR signal intensity (and several indirect supporting assays). Furthermore antioxidant supplementation should attenuate the post-exercise increase in plasma endotoxin if exercise-induced systemic endotoxaemia is free radical-mediated. There appears to be no reported evidence describing the role of the water-soluble antioxidant ascorbic acid in exercise-induced oxidative damage using ESR, or determining the effect of ascorbic acid supplementation on plasma endotoxin levels following maximal aerobic exercise. Although Goldfarb (1993) has suggested that ascorbic acid supplementation in exercise-induced oxidative stress needs to be examined.

1.1 AIMS AND OBJECTIVES.

The aim of this work is to apply the technique of ESR spectroscopy to the detection of free radical species in the blood of healthy exercising humans. Furthermore to provide additional supporting evidence using a range of indirect biochemical assays and also by examining the effect of antioxidant intervention on exercise-induced oxidative stress.

OBJECTIVES.

1. To develop an ESR method to apply to the detection of free radical increase in human serum following maximal aerobic exercise. This has not been previously demonstrated.

2. To examine the relationship between whole body oxygen uptake and free radical production. It is hypothesised that leakage of electrons from the mitochondrial electron transport chain will lead to increased free radical production during aerobic exercise. Study 1 (chapter 4) examines the role of maximal aerobic exercise in relation to free radical production and oxidative damage.

3. To determine the effect of anaerobic exercise on free radical production. There are no studies describing the effect of a Wingate anaerobic exercise test on free radical production in relation to ESR detection. The relative lack of increase in oxygen uptake during this type of exercise should prevent large increases in free radical production if mitochondrial electron transport chain leakage is the origin of the anticipated increase during aerobic exercise as previously suggested. This study (study 2, chapter 5) will allow conclusions to be drawn regarding the

mechanism and origin of any increase in free radical species observed during maximal aerobic exercise.

4. To perform an intervention study and attempt to modify exercise-induced oxidative damage by pre-treatment with antioxidants, specifically ascorbic acid. There are no studies reporting the effect of ascorbic acid supplementation on the attenuation of an exercise-induced increase in the ESR signal in human blood. Study 3 (chapter 6) examines the effect of antioxidant intervention on exercise-induced free radical production and oxidative damage.

5. Alongside studies 1 and 3, to identify the effect of exercise eliciting maximum oxygen uptake and antioxidant supplementation on plasma bacterial lipopolysaccharide (endotoxin) concentration. It is suggested that exercise-induced systemic endotoxaemia is free radical-mediated. Therefore supplementation with the antioxidant ascorbic acid should prevent this phenomenon by scavenging the free radicals produced by exercise.

6. Undertaking *in vitro* studies to attempt to identify the ESR signal and its origin.

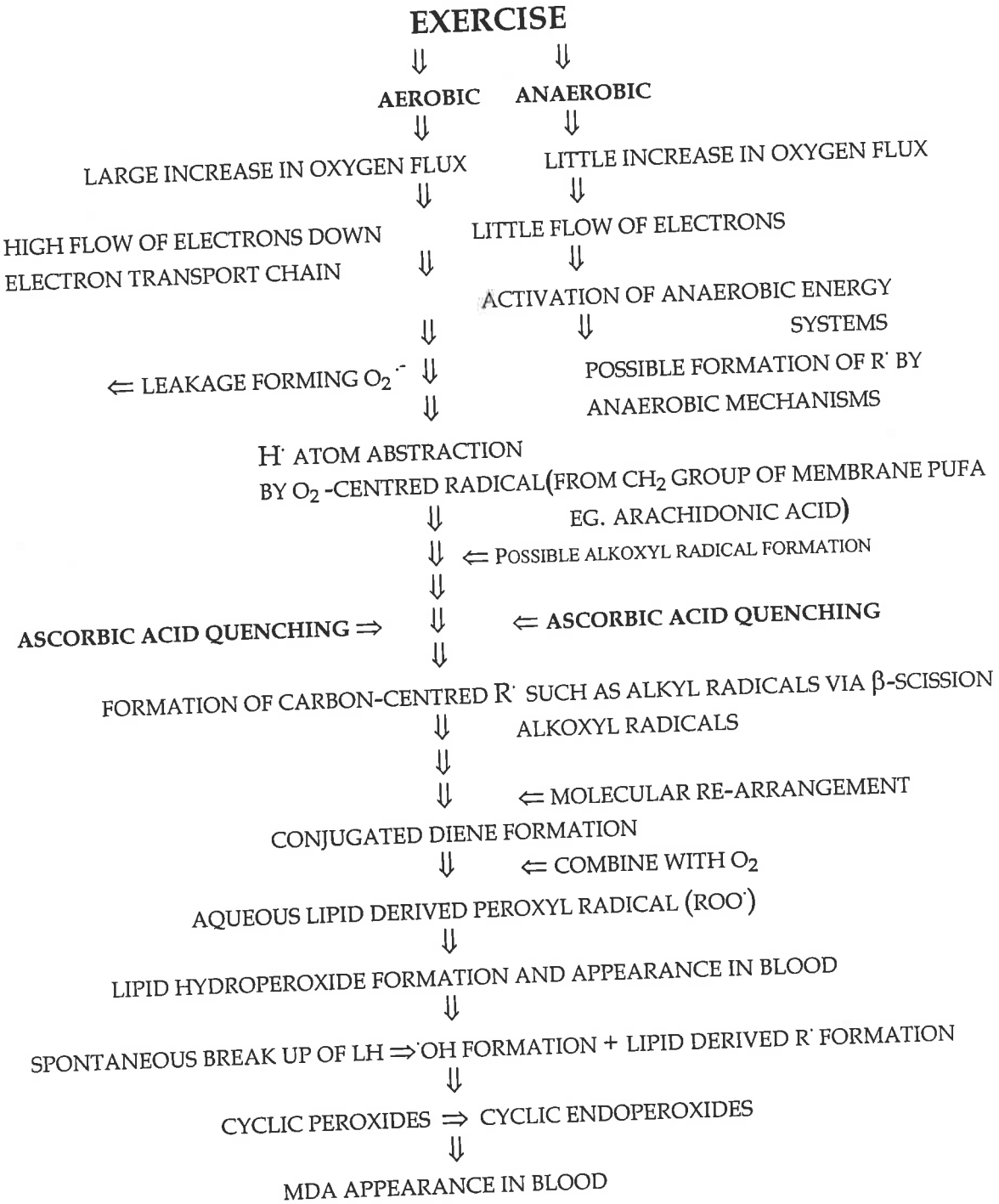
1.2 Statement of hypothesis.

The working hypothesis underpinning this thesis is that increased whole body oxygen flux and hence increased mitochondrial electron transport chain leakage results in increased free radical production leading to oxidative damage. The null hypothesis (H_o) is that aerobic exercise does not lead to increased free radical production or increases in the post-exercise ESR signal.

Therefore aerobic exercise, which elicits large increases in whole body oxygen uptake with concomitant increases in free radical production (as measured by ESR and several other indirect supporting indices of oxidative damage), should lead to significant increases in the post-exercise ESR signal intensity and the supporting indirect indices of oxidative damage. A test of this hypothesis is that an exercise test that is anaerobic in nature, and does not therefore cause a substantial increase in whole body oxygen uptake, should not cause an increase in the post-exercise ESR signal.

The implication is therefore that mitochondrial electron transport chain leakage is the origin of the anticipated increase in free radicals following aerobic exercise. During intense short-term anaerobic exercise, energy is supplied primarily by the breakdown of creatine phosphate and anaerobic glycolysis and is therefore not dependent on oxygen, increases in free radical concentration should not then occur. A biochemical model of this hypothesis is shown overleaf (Figure 1.0). Furthermore, if the post-exercise increases in the ESR signal intensity and indirect parameters of oxidative damage are free radical-mediated then antioxidant intervention should prevent these increases.

FIGURE 1.0: BIOCHEMICAL MODEL OF THE DEPENDENCE ON OXYGEN FLUX TO GENERATE INCREASED AMOUNTS OF FREE RADICALS DURING EXERCISE.



1.3 Overview of thesis.

This thesis is presented in seven main chapters as follows:

Chapter 2: Reviews the literature on free radical biochemistry *in vivo* with an emphasis on exercise and exercise-induced oxidative damage and free radical production. The effect of antioxidant intervention on exercise-induced oxidative stress and performance is also examined.

Chapter 3: General methodology, describes the theory of ESR spectroscopy, the development of the spin trapping extraction procedure and seven pilot studies. Blood sampling techniques and biochemical analyses are also described.

Chapter 4: Study 1 examined the role of oxygen uptake in relation to free radical production during exercise in humans. The study therefore examines aerobic exercise in relation to exercise-induced oxidative damage.

Chapter 5: Study 2 examines the role of anaerobic exercise in relation to exercise-induced free radical production.

Chapter 6: Study 3 is an antioxidant intervention study. The principle aim of this study was to examine the effect of the water soluble antioxidant ascorbic acid on exercise-induced free radical production under conditions of maximal whole body oxygen flux.

Chapter 7: *In vitro* studies. The aim of this work was to attempt to confirm the identity of the radical species detected by ESR in the human studies, and to attempt to determine their site of origin.

Chapter 8: Summarises and discusses the main findings of all studies.

Chapter Two

REVIEW OF THE LITERATURE

2.0 INTRODUCTION.

This chapter reviews the literature including possible mechanisms and pathways of free radical production *in vivo*, and seeks to draw together findings from research regarding these mechanisms to provide a basis for the human studies identified in section 1.1. It also reviews antioxidant intervention studies that have been performed in relation to exercise and exercise-induced oxidative damage.

The review of the literature is composed of 4 main sections:

Section 2.1: A broad introduction to free radicals and human biology.

Section 2.2: Describes the biochemistry and mechanisms of free radical production *in vivo*.

Section 2.3: Reviews the role of exercise in the generation of increased amounts of oxidants.

Section 2.4: Reviews pertinent antioxidant intervention studies.

2.1 FREE RADICALS AND HUMAN BIOLOGY.

Free radicals seem to have permeated much of current scientific endeavour, and it is virtually impossible to open a bio-medical journal without being confronted by at least one article on free radicals or oxidative stress. Along with this great interest and plethora of articles has arisen some controversy regarding the role free radicals play, for example in disease pathophysiology. Free radical metabolism is implicated in many disease states, however what is not clear is the

precise role played by these often highly reactive compounds. What is quite certain is that increased free radical production will occur as a result of tissue injury, but there is uncertainty as to whether or not these species cause the initial tissue injury. Professor Trevor Slater has eloquently summed up this conundrum by stating an answer to this very question. In an article entitled; "Free radical disturbances and tissue damage: Cause or consequence?" He states the answer as being: "it depends" (Slater 1988).

The term 'oxidative stress' has been coined to describe free radical involvement in disease, and may be more specifically defined as a condition where the *in vivo* antioxidant and pro-oxidant reactions shift in favour of pro-oxidants.

Oxidative stress may be mediated by the following mechanisms:

1. Increased activity of radical-generating enzymes, eg. xanthine oxidase;
2. Activation of phagocytes;
3. Activation of phospholipases, cyclooxygenases and lipoxygenases;
4. Dilution and destruction of antioxidants;
5. Release of 'free' metal ions from sequestered sites and/or muscle;
6. Release of haeme protein eg. haemoglobin, myoglobin;
7. Disruption of electron transport chains and increased electron leakage for superoxide anion $O_2^{\cdot -}$

(Aruoma 1994).

Importantly these activities could well be amplified by toxins, hyperoxia and exercise (Aruoma 1994).

Given the nature of free radical reactions *in vivo*, initial or primary reactions such as hydrogen atom abstraction (initiation) from the cell lipid bi-layer, or superoxide formation leading to hydrogen peroxide accumulation, can lead to secondary reactions and a build up of reaction products eg. conjugated dienes and lipid hydroperoxides. These in turn can cause further cell damage and also perhaps more importantly further free radical production (propagation), so that one radical begets another. However, initial injury may also lead to repair of the site of damage, or scavenging by low molecular weight antioxidants such as ascorbic acid (termination). For example the appearance of thymine and thymidine glycol in the urine is thought to indicate DNA damage and repair (Cathcart et al 1984). Thus if so many free radicals are produced that the antioxidant defences are overwhelmed or, they do not work efficiently then oxidative stress is likely and free radicals will initiate tissue damage.

Increased production of free radicals have been implicated not only in disease but also in exercise, particularly where oxygen consumption is increased (Alessio and Goldfarb 1988, Alessio et al 1988, Kanter et al 1986, Kanter et al 1988, Salminen and Vihko 1983). The difficulty arises in determining which occurs first, increased free radical production or tissue damage. Unfortunately, the lack of suitable assays has greatly impeded free radical research and a definitive answer is still not possible. The lack of such assays is primarily due to the transient nature of free radicals and their reaction products, low steady state concentration, coupled with the complex biochemistry of human subjects in particular. Analytical methods however continue to advance and developments in ESR and spin trapping technology will help elucidate the precise role free radicals play in both the disease and exercise model.

A free radical may be defined as any species capable of independent existence that possesses an unpaired electron an unpaired electron is one that is alone in an

atomic or molecular orbital (Halliwell and Gutteridge 1989). The presence of the unpaired electron causes the species concerned to exhibit paramagnetism - the property of reacting to an applied magnetic field - which is the basis for detection by electron spin (paramagnetic) resonance spectroscopy. The unpaired electron also causes the species to be highly reactive. A free radical, by convention, is indicated by the application of a superscript dot ($^{\bullet}$) designating the presence of the unpaired electron.

Free radicals may be positive, neutral or negatively charged. Positive radicals are produced in a mass spectrometer chamber when electrons collide with organic molecules to displace valence electrons. However, for the purposes of this thesis only radicals which are of biological significance will be discussed, and these tend to be neutral or negatively charged eg. superoxide anion ($O_2^{\bullet -}$).

The polarity of a radical is important in that superoxide does not enter cells readily but may do so via the negatively-charged chloride (Cl^-) channel as in an erythrocyte, for example. This is compared to hydrogen peroxide (H_2O_2) which may readily diffuse across cell membranes. Importantly there is some speculation that this is how hydrogen peroxide exerts its damaging effects on such critical cellular targets as oxidation of the nuclear regulatory protein NFkB (Halliwell and Gutteridge 1989, Schreck et al 1991). The highly reactive hydroxyl radical may be produced *in vivo* by reaction of transition metal ions and homolytic fission of water which may intitiate a chain reaction of free radical formation. There are many different kinds of radical, including oxygen-, carbon - and nitrogen-centred radicals, many of which are produced to a greater or lesser degree in the body.

2.2 THE BIOCHEMISTRY OF FREE RADICAL PRODUCTION - *IN VIVO.*

Free radicals are continually produced *in vivo* during resting energy metabolism (Alessio 1993). It is pertinent at this point to introduce the concept of Reactive Oxygen Species (ROS), since there are other products generated in the body that exhibit some of the potentially toxic properties of free radicals, but which are not free radicals themselves, such as hydrogen peroxide. Human beings are dependent on oxygen for survival, and it is this very dependence on the reduction of oxygen to water that may cause increased ROS production.

During the various stages of the univalent reduction of molecular oxygen to water, different free radicals and other toxic oxidants may be produced (see figure 2.0). One electron reduction results in the production of the superoxide anion ($O_2^{\cdot -}$). Two electron reduction results in hydrogen peroxide (H_2O_2) production, which using the previous definition is not a free radical but is a ROS. Three electron reduction yields the most reactive free radical, the hydroxyl radical ($^{\cdot}OH$), which is so reactive that it reacts at almost diffusion-controlled rates with any molecule that it comes into contact with and thus causes damage at or near its site of generation. This concept of site specificity is important in the biological significance of the hydroxyl radical since if this radical is formed near DNA then it may damage the DNA backbone or pyrimidine base. DNA damage is thought to be an early event in oxidative stress.

Figure 2.0: Univalent reduction of oxygen and consequent radical formation.

$O_2 \xrightarrow{e^-} O_2^{\cdot-}$ One electron reduction yielding superoxide anion

$2O_2^{\cdot-} \xrightarrow{e^- + 2H^+} H_2O_2 + O_2$ Two electron reduction

$H_2O_2 \xrightarrow{e^- + H^+} {}^{\cdot}OH + OH^-$ Three electron reduction yielding hydroxyl radical

${}^{\cdot}OH \xrightarrow{e^- + H^+} H_2O$ Four electron reduction yielding water

In addition to the above-mentioned species singlet oxygen ($^1O_2$) and hypochlorous acid (HOCl) may be formed, which are also examples of ROS. In the context of exercise physiology ROS's are of particular interest and are of biological significance. The above species are all related to oxygen and thus a question may be posed: is oxygen toxic?

Antioxidant defence mechanisms *in vivo* are legion and thus would suggest that the answer to the question is yes. The production of superoxide and hydrogen peroxide is regulated by the enzymes superoxide dismutase and catalase, respectively, thus suggesting the need to control these ROS. The superoxide theory of oxygen toxicity (McCord and Fridovich 1969, Fridovich 1975), states that the formation of the superoxide radical *in vivo* plays a major role in the toxic effects of oxygen.

Other free radicals that are not oxygen-centred are also continually produced in the body, the most notable of which is nitric oxide ($NO^{\cdot}$) in which the unpaired electron is de-localized between both atoms (Halliwell et al 1992). Nitric oxide is abundant in the body and is known to exert a relaxing effect on the vascular endothelium and is therefore known as endothelium-derived relaxing factor (EDRF) (Zweier 1995). Its interaction with superoxide anion to form peroxy-nitrite ($ONOO^{\cdot-}$) may have important implications during exercise since by opposing or inhibiting the action of $NO^{\cdot}$, $O_2^{\cdot-}$ may act as a vasoconstrictor. In addition to this $ONOO^{\cdot-}$ can lead to the production of $^{\cdot}OH$ radical thus causing endothelial injury via the mechanism shown below:

FIGURE 2.1: Interaction of $O_2^{\cdot-}$ and $NO^{\cdot}$ leading to $ONOO^{-}$ formation.

$$O_2^{\cdot-} + NO^{\cdot} \Rightarrow ONOO^{-} + H^{+} \Leftrightarrow ONOOH \Rightarrow HO^{\cdot} + NO_2^{\cdot} \Rightarrow NO_3^{-} + H^{+}$$

(after Beckman et al 1990).

2.2.1 MECHANISM OF FREE RADICAL PRODUCTION *IN VIVO*.

MITOCHONDRIAL ELECTRON TRANSPORT CHAIN.

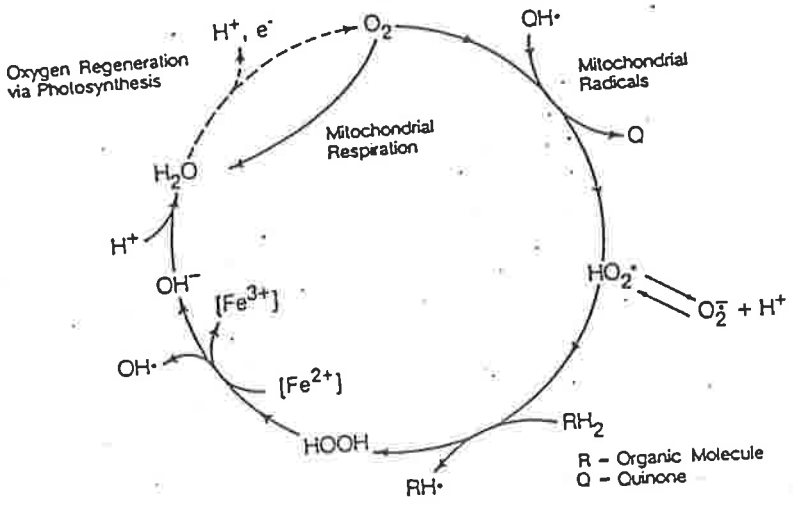
The site of oxygen reduction in mammalian cells - the powerhouse of the cell - is the mitochondrion. Mitochondria are ubiquitous throughout the body and play a central role in free radical formation. Probably the most important sources of $O_2^{\cdot-}$ *in vivo*, in most aerobic cells are the electron transport chains of the mitochondria and endoplasmic reticulum (Halliwell and Gutteridge 1989).

Muscular exercise is dependent upon the conversion of chemical bond energy to mechanical energy. High-energy phosphate bonds in adenosine tri-phosphate (ATP) is the energy currency that the muscle fibres use to contract. Since stores of ATP are limited it must be continuously regenerated through a number of metabolic pathways, including: anaerobic glycolysis; oxidation of carbohydrate and fatty acid intermediates in the Krebs cycle; and the mitochondrial electron transport chain which accounts for over 80% of ATP production (Halliwell 1994).

Electrons are transferred from Krebs cycle substrates directly from succinate or the electron carrier NAD^+. These electrons pass through a sequence of protein and non-protein carriers to the catalytic site of cytochrome oxidase where four electrons together with four hydrogen ions reduce molecular oxygen to water (tetravalent reduction). The critical site for oxygen radical formation is the reduced semiquinone located in the space between the NADH-CoQ complex or succinate dehydrogenase and cytochrome b (Sjodin et al 1990). The most efficient pathway for ATP regeneration is via oxidation of local glycogen and fat stores in the musculature. The terminal point of this oxidation process requires molecular oxygen as an electron acceptor and proceeds in the Krebs cycle and mitochondrial electron transport chain. The oxygen cycle/pathway is presented in figure 2.2 and the pathway for oxygen transport and utilisation is presented in figure 2.3 below where VCO_2 and VO_2 are the dynamic volumes of carbon dioxide and oxygen respectively.

It can be seen from these figures that there is a possibility of leakage of electrons from the mitochondria resulting in the formation of both quinone radicals as well as the highly reactive hydroxyl and superoxide radicals. Furthermore figure 2.3 shows the centrality of mitochondria in oxygen useage and energy production *in vivo*.

Figure 2.2: THE PATHWAY FOR OXYGEN.

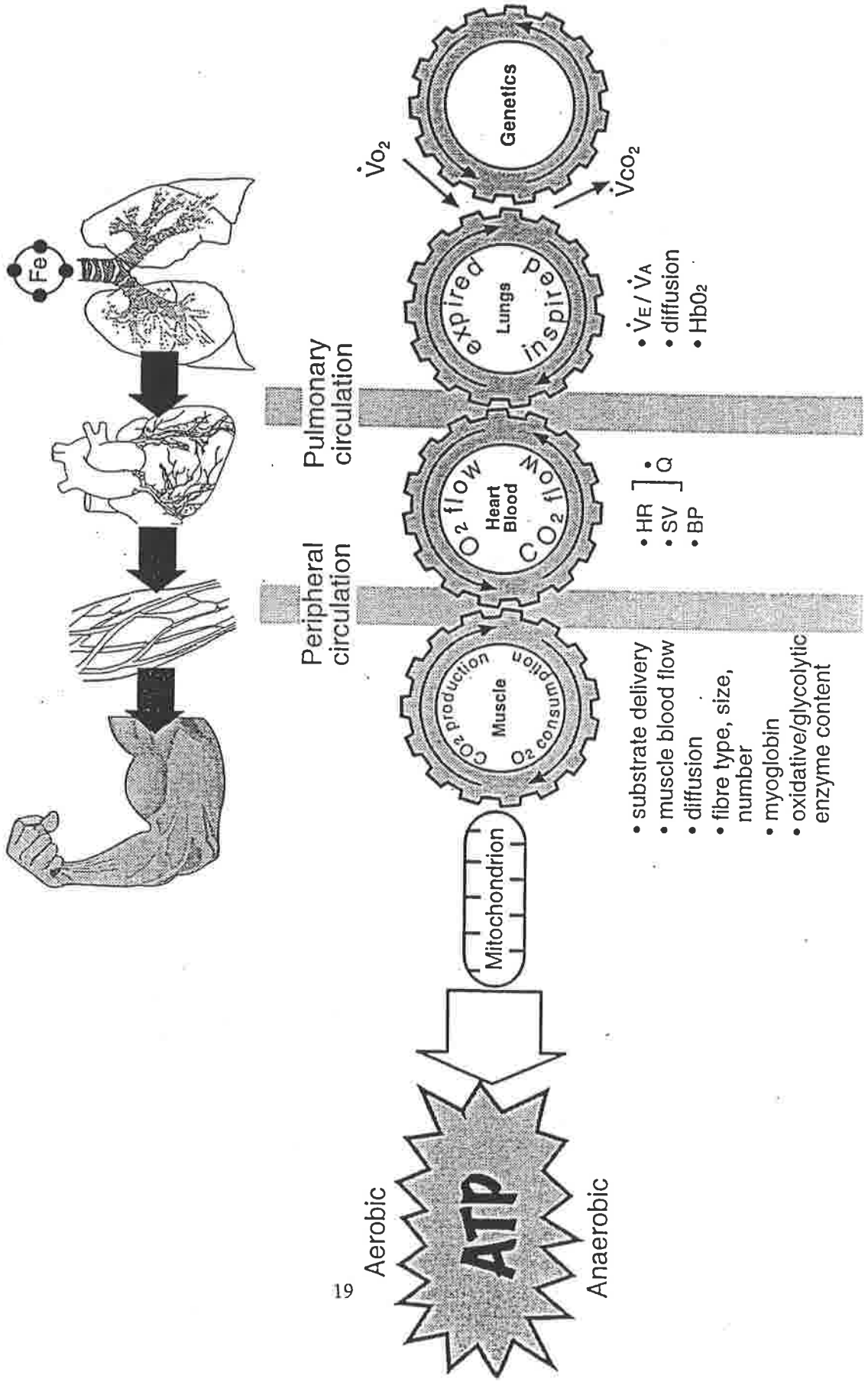


(Packer 1986).

Since exercise, particularly that of an aerobic nature, causes large increases in whole body oxygen consumption then it is logical to assume that this causes an increase in electron flow down the mitochondrial electron transport chain. Whole body oxygen uptake can rise 15 to 20 fold above resting levels during strenuous aerobic exercise and as much as 50-fold in active skeletal muscle (Newsholme et al 1994).

Some of the electrons that pass down the respiratory chain may leak to form potentially harmful ROS. Estimates of percent leakage vary: from 1% to 3% (Halliwell 1994) to between 2% and 5% (Boveris and Chance 1973) of the total electron flux. Sawyer (1988) has suggested that the percentage of electrons leaked may actually be as high as 15%. Thus it is distinctly possible that increases in electron flux through rapidly respiring mitochondria can lead to an enhancement of electron leakage and consequent ROS production as a direct result of exercise.

Figure 2.3: OXYGEN TRANSPORT AND UTILISATION (modified from Wasserman 1987).



Not only is electron leakage important, it is estimated that for every 25 molecules of oxygen reduced by cytochrome oxidase, one oxygen molecule is reduced by semi-quinone to form a free radical (McCord 1979). The production of superoxide in the mitochondrial membrane is thought to be the precursor for the hydrogen peroxide observed in mitochondria (Boveris and Cadenas 1975). The rate of hydrogen peroxide formation in mitochondria is directly related to the energy coupling mechanism (Loschen et al 1973). Thus the greater energy demand occurring during exercise causes enhanced electron leakage, increased ROS production and concomitant exercise-induced oxidative stress.

XANTHINE OXIDASE.

Xanthine exists primarily in two enzymatic forms *in vivo*: xanthine dehydrogenase and xanthine oxidase. Xanthine dehydrogenase is located primarily in the vessel walls of tissues, notably cardiac and skeletal muscle. The oxidase form is frequently used as a source of superoxide in experiments *in vitro* (Halliwell and Gutteridge 1989), and has been shown to produce not only superoxide but also the hydroxyl radical together with singlet oxygen, hydrogen peroxide and lipid peroxidation via a xanthine oxidase system (Kellogg and Fridovich 1975). However, the vast majority of this enzyme *in vivo* exists as the dehydrogenase form.

The function of xanthine dehydrogenase as it participates in purine metabolism is to transfer electrons to nicotinamide adenine dinucleotide (NAD^+). It does this as it oxidizes xanthine to hypoxanthine and hypoxanthine to uric acid (Hellsten 1994). If tissue homeostasis becomes disrupted, for example during ischaemia, then the dehydrogenase may be converted to the oxidase form by oxidation of the essential sulphydryl (-SH) groups or by proteolysis (Halliwell and Gutteridge

1989). Oxidation of the -SH groups may occur in response to lowered cellular thiol status, including glutathione, which occurs in ischaemia.

Xanthine oxidase uses molecular oxygen as an electron acceptor. The univalent reduction of oxygen catalysed by xanthine oxidase leads to the formation of the superoxide radical (McCord and Fridovich 1969).

During intense exercise it is distinctly possible that tissues may become transiently ischaemic and thus xanthine oxidase may become activated and generate superoxide radicals. However, this has recently been disputed since it is suggested that unlike "typical" ischaemia, such as would occur in a myocardial infarction, oxygen is always present during exercise-induced oxidative stress (Sjodin et al 1990). Whilst this is certainly possible, during intense exercise hypoxia is known to occur in certain tissues, such as the kidney and gut, as blood is shunted away to the working muscles, and even at lower exercise intensities skeletal muscle fibres may themselves experience hypoxia (Witt et al 1992). The mucosal villi of the intestine contain the greatest concentration of xanthine dehydrogenase *in vivo* (McCord 1985). This may be important for the research contained in this thesis since hypoxia may cause xanthine oxidase to be formed in the gut leading to superoxide formation and tissue damage thus allowing translocation of endotoxin into the circulation.

Following cessation of exercise, reperfusion and thus re-oxygenation will occur causing the associated burst of ROS formation and possible tissue damage (Wolbharst and Fridovich 1989). ESR evidence for the generation of reactive oxygen species following ischaemia /reperfusion has been provided from the study of oxygen-derived free radical generation during myocardial ischaemia and reperfusion in male Sprague-Dawley rats (Baker et al 1988). The results clearly implicated the formation of the superoxide radical and the authors

suggested the site of production as being between the ubiquinone and cytochrome b, located on the inner mitochondrial membrane. Raised plasma levels of hypoxanthine have been shown to occur following brief intense exercise in human subjects and it is suggested that this originates from the working muscles (Sahlin et al 1991).

Central to the generation of superoxide radicals is the maintenance of cellular ATP levels. During "typical" ischaemia ATP may not be regenerated due to lack of oxygen, whereas exercise-induced ischaemia may cause low levels of ATP because of the high rate of ATP turnover, ie. utilisation of ATP exceeds re-generation. The end point of this situation would be a decrease in the amount of available ATP which could severely disturb cellular homeostasis possibly leading to increased free radical production and oxidative stress.

THE ROLE OF CALCIUM IN FREE RADICAL GENERATION.

Hypoxia may cause depletion of ATP which in turn may be insufficient to allow ATP-dependent calcium pumps to continue functioning (Sjodin et al 1990). Malfunction of these calcium pumps can lead to intracellular calcium overload which has been implicated in the mechanisms of cellular damage and exercise-induced oxidative stress (Jackson 1994). This may also lead to activation of calcium-dependent proteases. Activation of these proteases causes a cleavage of a peptide from xanthine dehydrogenase leading to a change in the conformation of the enzyme to xanthine oxidase (Sjodin et al 1990). As mentioned xanthine oxidase uses molecular oxygen as an electron acceptor instead of NAD^+, which is then reduced forming the superoxide radical (McCord 1985).

Raised intracellular calcium may be particularly important in exercise-induced oxidative stress since it may be an inevitable consequence of excess contractile

activity. Stimulation of mouse muscle under hypoxic conditions has been shown to result in muscle damage accompanied by accumulation of calcium and concomitant efflux of degradative enzymes (Claremont et al 1984). The significance of this lies in the role calcium plays in free radical-induced cellular damage and necrosis. Following a net influx of extracellular Ca^{2+} down the large extracellular-intracellular concentration gradient for this element, cytosolic enzyme efflux from damaged skeletal muscle and ultrastructural changes occur, with activation of degenerative pathways such as activation of phospholipase (Jackson 1990).

Activation of phospholipase enzymes such as phospholipase A_2, leading to calcium-induced membrane phospholipid hydrolysis is an important stage in the mechanism leading to efflux of these intracellular enzymes (Jackson 1990). One possible mechanism for this is that phospholipase activation leads to prostaglandin and creatine kinase release from muscle, and that the calcium accumulation seen in damaged muscle induces release of arachidonic acid from membrane phospholipids via the activation of phospholipase A_2, thus providing the substrate for increased prostaglandin production which is a recognised pathway for free radical production in skeletal muscle (Jackson 1990, Jackson et al 1987, Jackson et al 1984). Studies of menadione (2-methyl-1,4-napthoquinone) induced cell damage have helped elucidate the role calcium plays in free radical-induced oxidative stress. Menadione metabolism involves the one electron reduction of the quinone which results in the formation of the semi-quinone radical, this can rapidly reduce molecular oxygen, forming the superoxide radical and regenerating the parent quinone. Dismutation of superoxide results in hydrogen peroxide formation and consequent hydroxyl radical production in the presence of transition metal ions (Halliwell and Gutteridge 1989), and can quickly lead to oxidative stress as redox cycling of the quinone continues.

Menadione inhibits plasma membrane Ca^{2+}-ATPase activity through oxidation of protein thiols, thus causing release of Ca^{2+} from intracellular stores and preventing sequestration and extrusion of Ca^{2+} from the cell, leading to sustained increase in the concentration of cytosolic Ca^{2+}, a condition which results in cytotoxicity (McConkey and Orrenius 1988). It is clear that the structural changes and the disruption of Ca^{2+} homeostasis associated with oxidative stress are important components of free radical induced cellular damage (Jackson and Edwards 1986), however a cause and effect relationship is difficult to establish. Free radicals therefore invoke a variety of intracellular changes, including perturbations of thiol and Ca^{2+} homeostasis which may be associated with oxidative stress.

ACTIVATED PHAGOCYTES.

Phagocytes are cells that are able to engulf and digest bacteria and include macrophages and activated neutrophils. Macrophages are released from bone marrow as immature monocytes and mature in various tissues where they may reside for anything from weeks to years (Reeves and Todd 1993). Macrophages have considerable ability to secrete cytokines such as interleukin 1, interferon and prostaglandins such as PGE_2 and $PGF_{2\alpha}$, themselves a potent source of ROS (Reeves and Todd 1993).

Polymorphonuclear neutrophil leucocytes mature and are stored in bone marrow, they are released rapidly into the circulation in response to various stimuli, most notably bacterial infection but also possibly strenuous exercise, and have the capacity to produce relatively large amounts of reactive oxygen species (Reeves and Todd 1993). Phagocytes contain both oxygen-dependent and oxygen-independent anti-microbial mechanisms. For example, lysozyme

hydrolyses the peptidoglycal of gram $+^{ve}$ cell walls, while the bacteriostatic effect of lactoferrin is related to its ability to bind iron strongly. Perhaps the most notable aspect of phagocytosis that occurs is that it is accompanied by a burst of respiratory activity initiated by a membrane oxidase, thought to be NADPH oxidase, which reduces molecular oxygen to the superoxide anion (Reeves and Todd 1993).

Figure 2.4: Sequential production of cytotoxic oxygen compounds in phagocytic cells.

1. $O_2 \xrightarrow[+e]{\text{membrane oxidase}} O_2^{\cdot -}$

2. $2O_2^{\cdot -} + 2H^+ \xrightarrow{\text{spontaneous}} H_2O_2 + {}^1O_2$

   or, $\xrightarrow{\text{SOD}} H_2O_2 + {}^1O_2$

3. $H_2O_2 + O_2^{\cdot -} \longrightarrow {}^{\cdot}OH + OH^- + {}^1O_2$

4. $H_2O_2 + Cl^- \xrightarrow{\text{MPO}} HOCl^- + OH^-$

5. $OCl^- + H_2O_2 \longrightarrow {}^1O_2 + Cl^- + H_2O$

SOD = Superoxide dismutase

MPO = Myeloperoxidase (Adapted from: Reeves and Todd 1993).

Much of the respiratory activity takes place within the hexose monophosphate shunt which provides NADPH as an electron donor for the reduction of molecular oxygen with the concomitant production of superoxide (Reeves and Todd 1993). This process, which is initiated at the cell surface, continues on the inner surface of the phago-lysosome where superoxide is converted to antimicrobial hydrogen peroxide by spontaneous dismutation primarily at the cell surface, with the production of singlet oxygen (Reeves and Todd 1993). The reaction may also be driven by superoxide dismutase which is present intra-cellularly giving rise to molecular oxygen (as shown in figure 2.4).

Singlet oxygen is a highly reactive and unstable molecular species which has light-emitting properties as it returns to ground state, thus the technique of chemiluminescence is used in the study of free radicals. While hydrogen peroxide and superoxide can also interact to form the most reactive oxygen free radical, the hydroxyl radical.

A major source of microbicidal activity develops in the phago-lysosome when hydrogen peroxide interacts with halide (Cl^- in the neutrophil and I^- in the macrophage) in the presence of myeloperoxidase to form hypohalite and water. Hypohalite can then further react with hydrogen peroxide to form more singlet oxygen. It is clear that a plethora of toxic material is produced during the respiratory burst, and that the deliberate production of superoxide is central to the bactericidal action of phagocytes (Babior 1978).

It is also known that human fibroblasts release reactive oxygen species upon exposure to synovial fluid from patients with a free radical mediated disease, such as rheumatoid athritis (Meier et al 1990). In the study ESR evidence suggested that superoxide was the primary radical formed. Low level-chemiluminescence was also employed to detect light emitted from activated

phagocytes. It is worth noting however, that there are a number of processes that limit the spread of these toxic metabolites.

For example, catalase, largely present in peroxisomes, converts hydrogen peroxide to water and oxygen, while superoxide dismutase converts superoxide to hydrogen peroxide. Hydrogen peroxide is also broken down by the glutathione redox system involving glutathione peroxidase.

Another aspect of the immune response is redistribution of iron. This may well be a double-edged sword, since although bacteria do not grow well in an iron-deficient medium (Kluger and Rothenburg 1979), any increase in intracellular iron where superoxide and hydrogen peroxide are found could promote the formation of the hydroxyl radical from the Fenton reaction. Alternatively movement of ROS across cell membranes which hydrogen peroxide is able to do, may well lead to it coming into contact with iron. This would also potentially lead to hydroxyl radical formation since iron catalyzes the formation of the hydroxyl radical from superoxide and hydrogen peroxide via Fenton chemistry (Halliwell and Gutteridge 1990).

Perhaps the most notable aspect of the previously described immune response is its specificity. However, infection and/or trauma induce not only specific immune reactions in response to the recognition of a specific antigen, but also non-specific host defence responses that are collectively known as the acute phase response. The acute phase immune response is mediated through release of cytokines and results in: proliferation of white cells (leukocytosis); fever; iron redistribution and contributes to the production of ROS during exercise (Cannon and Blumberg 1994).

Other cells of the immune system -notably lymphocytes- can also cause cytotoxicity and inflammation. ROS production by immunological cells has been observed in activated phagocytes and more recently, B lymphocytes (Maly 1990). However, phagocytic cells of virtually every species use molecular oxygen to form superoxide (Styrt 1989).

Formation of superoxide will inevitably lead to hydrogen peroxide production and possibly other ROS, the most notable of which is the hydroxyl radical (Klebanoff 1988). ESR, in conjunction with a spin trapping technique, has been used to determine hydroxyl radical involvement in the inflammatory behaviour of human mononuclear phagocytes (Cohen et al 1991). Results suggest the hydroxyl radical may play an important role in the inflammatory behaviour of human phagocytic cells.

There is an increased prevalence of infection in athletes undertaking strenuous exercise, particularly upper respiratory tract infection (Nieman 1994). Therefore perturbations of the immune system may be an important source of ROS during and following exercise of a strenuous nature and/or long duration. This is borne out by the fact that there is a marked increase in the total number of circulating leukocytes immediately following a marathon, and that the cells of the immune system may be less able to mount a defence against strenuous or prolonged exercise (Castell et al 1996).

In addition to this downhill running which involves predominantly eccentric muscle contractions, has been shown to induce a greater mobilization of leukocytes and neutrophils in comparison to level running (Pizza et al 1995), suggesting that eccentric exercise is associated with acute inflammation and an immune response.

MECHANICAL MECHANISMS AND MUSCULAR CONTRACTION.

Excessive exercise involving high mechanical stresses (eg. long distance running) or contact sports (eg. rugby football) may result in bleeding. Resultant leakage of erythrocyte contents, such as haemoglobin, potentially provides a ready source of transition metal ions to participate in free radical generating reactions. This bleeding may be caused by heel strike, (so-called heel strike haemolysis), or the heavy contact of a rugby tackle causing a haematoma.

In addition to these mechanisms, of particular importance is the type of contractile activity which the muscle undertakes since this appears to greatly influence the extent of muscle damage produced (Jackson and O'Farrell 1993). Eccentric muscle contraction where the muscle is lengthened during contraction (as would occur during downhill running, box stepping or cycling), can lead to excessive shear or metabolic stresses which are distinct from the mechanical stress imposed by other types of exercise. These exercise types may exert a greater mechanical cost which leads to muscle damage and perturbations in cellular homeostasis, causing the release of muscle-derived enzymes, such as creatine kinase.

In terms of oxygen requirement the demand during eccentric contractions is low when compared to whole body dynamic exercise where a mixture of concentric and eccentric contractions are seen. This implicates mechanisms other than mitochondrial leakage as a source of ROS production during predominantly eccentric exercise.

Tissue damage and free radical formation may well be synonymous. Whilst muscle damage and soreness following un-accustomed or exhaustive exercise is a well recognised phenomenon, and large amounts of creatine kinase are

commonly seen in blood plasma during and after such exercise (Jackson and Edwards 1986).

Muscle damage and/or soreness following exercise can be divided into two categories:

i. That which occurs within twenty four hours of exercise.
This is commonly attributed to concentric muscle contraction.

ii. That which is delayed to forty-eight or seventy-two hours post-exercise. This is frequently known as delayed onset muscle soreness (DOMS) and may be attributed to the greater metabolic stress imposed by eccentric muscle contraction.

Initial damage is followed by inflammation where it is suggested that calcium plays an important role in triggering the inflammatory changes (McArdle et al 1992), since loss of calcium homeostasis affects muscle cell viability (Jones et al 1984). This leads to calcium influx and accumulation from the extracellular fluid (Kuipers 1994).

Activation of calcium-dependent degenerative pathways have been implicated in skeletal muscle damage (Jackson et al 1984). This is supported by further evidence using ESR implicating free radical involvement in damage induced by electrically-stimulated muscle contraction (Jackson et al 1985). Notably a stable ESR signal occurs following treatment with a calcium ionophore (A23187) which causes a loss of calcium homeostasis (Johnson et al 1988).

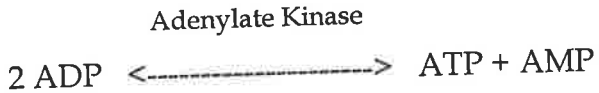
Thus, exercise-induced muscle damage probably involves not only oxygen-dependent free radical mediated pathways but also calcium- dependent

degenerative processes and mechanical stress. However, there are still many unanswered questions regarding the precise patho-physiological mechanisms.

ADENYLATE KINASE REACTION.

Under conditions of ADP accumulation (or ATP depletion) as would occur in anaerobic exercise, for example, the anaerobic lactate-forming pathways are utilised to provide energy for muscular work. The resulting accumulation of hydrogen ions and ADP leads to the activation of the adenylate kinase system. Adenylate kinase catalyses the conversion of two moles of ADP to one mole of ATP and AMP respectively (see figure 2.4).

Figure 2.5: Adenylate kinase reaction.



(Sjodin et al 1990)

The activation of the adenylate kinase system leads to the formation of hypoxanthine, which is a substrate for the enzyme xanthine oxidase located in the capillary endothelial cells catalysing the formation of uric acid from hypoxanthine and, using molecular oxygen as electron acceptor, forms superoxide radicals (Sjodin et al 1990), as described previously.

PROSTANOID METABOLISM.

The metabolism of prostanoids provides a ready source of ROS *in vivo* (Halliwell and Gutteridge 1989). Arachidonic acid, a precursor of prostaglandins, yields

reactive metabolites via the action of lipoxygenase enzymes. Thus the arachidonic acid cascade is a recognised source of oxygen radical production *in vivo*.

The significance of this pathway during exercise lies with the fact that inflammation and soreness resulting from strenuous or un-accustomed exercise, results in the activation of the arachidonic acid cascade producing prostaglandins and concomitant free radical production. Prostaglandins and serum lipid peroxides/malondialdehyde have been shown to increase following an acute bout of maximal cycle ergometer exercise (Viinikka et al 1984).

OTHER MECHANISMS.

Other mechanisms exist that can lead to the formation of ROS, most notably the hydroxyl radical. Transition metal ions such as iron and copper, play a central role in the Fenton reaction below. While hydrogen peroxide is strongly implicated in the Haber-Weiss reaction yielding hydroxyl radical. Hydrogen peroxide is produced *in vivo* by the action of superoxide dismutase.

Figure 2.6: Reactions of hydrogen peroxide causing ROS production.

i. Iron-catalysed Fenton reaction.

$$Fe^{2+} + H_2O_2 \text{ ----------> } Fe^{3+} + HO^- + {}^{\cdot}OH$$

ii. Haber-Weiss reaction.

$$O_2 + H_2O_2 \text{ ----------> } O_2 + HO^- + {}^{\cdot}OH$$

(Aruoma 1994).

Thus there are identified pathways by which these damaging ROS are produced *in vivo*. Although it is debatable as to whether Fenton chemistry occurs *in vivo* since transition metals are tightly bound to storage and transport proteins such as ferritin and transferrin respectively, possibly preventing their participation in free radical-generating reactions.

Aruoma and Halliwell (1991) suggest that transition metal ions are unavailable to catalyse reactions in human plasma. For example iron ions bound to transferrin and copper ions bound to caeruloplasmin or albumin cannot stimulate lipid peroxidation or $^{\cdot}OH$ production in human plasma, and furthermore caeruloplasmin-bound copper does not accelerate free radical reactions (Gutteridge and Stocks 1981, Aruoma and Halliwell 1987, Halliwell and Gutteridge 1990, Halliwell and Gutteridge 1989). It is worth noting however that low temperature storage of plasma samples may lead to release of caeruloplasmin-bound copper and could feasibly lead to artifactual results (Halliwell and Gutteridge 1989).

2.3 Exercise and Free Radical Production.

In recent years there has been an explosion of interest in exercise-induced free radical production. Many studies have been carried out purporting to demonstrate exercise-induced oxidative stress, and these will be discussed later. A distinction must however be drawn between an increase in free radicals brought about by exercise and oxidative stress. Oxidative stress will occur when the antioxidant defence mechanisms are overwhelmed by the abundance of oxidants in the system or when the antioxidant defences are compromised (for example by diseases such as diabetes mellitus or poor dietary intake of nutritional antioxidants). The results from the various studies have been

somewhat contradictory, although this can be legitimately explained by the wide variation in methods used to detect free radicals and the wide variation in exercise protocols adopted in the studies. There is however, a clear trend in the literature that reveals a consistent demonstration of increased free radical production following strenuous or exhaustive exercise. This will now be explored.

2.3.1 Electron Spin Resonance (ESR) evidence of exercise-induced free radical production.

Despite the large body of literature that now exists on free radical metabolism and exercise, extremely few studies have been carried out using the only method that can directly detect free radical presence, namely ESR spectroscopy. The studies that have used ESR spectroscopy have relied on the animal model. There appear to be no studies applying the technique to the measurement of exercise-induced increases in free radical concentration in the human venous circulation in any setting. The few studies that have used ESR in an exercise setting will now be reviewed.

Davies et al (1982).

The first and arguably the most widely quoted study using ESR spectroscopy was performed by Davies et al (1982). The study examined free radical production produced by exercise in male Long Evans rats (n = 6). Two types of exercise protocol were used in this study:

i. A progressive sub-maximal work intensity endurance test in which time to exhaustion was measured.

ii. A progressive intensity exercise test designed to elicit the maximum work capacity of the animals.

The second of the two tests is analogous to the progressive maximal oxygen uptake test designed to elicit maximal oxygen uptake and when continued to exhaustion, maximum work capacity.

In addition to measuring free radical production in a control group of rats fed standard laboratory chow, another group of rats were assigned a vitamin E-deficient diet. Rat gastrocnemius, soleus and plantaris together with liver homogenates, were analysed for free radical production at room temperature using a Varian E 109 spectrometer. In order to eliminate the possibility of artifactual results washed intact liver tissues were analysed as well as tissue homogenates.

However, the absence of whole muscle ESR spectra may give cause for concern, since it is possible to demonstrate an ESR signal in whole muscle tissue, at 77 K, following muscular contraction (Jackson et al 1985). Also the spectrometer gain is not reported for any of the analyses, although in fairness the authors state that all other conditions were kept constant. Results indicated a two to three-fold increase in the ESR signal intensity in muscle and liver homogenates of rats following the sub-maximal workload test to exhaustion, results for the progressive test are not reported. A g-value of $\cong$ 2.004 was ascribed to the ESR signal which compares favourably to that reported later by Jackson et al (1985).

The ESR signal strengths were consistently greater in tissues that had been very active. The authors speculate that exercise causes increased rates of ubisemiquinone turnover in mitochondrial electron transport chain and/or haemoglobin auto-oxidation, leading to increased rates of superoxide

production. It is worth noting that an ESR signal was clearly visible at basal conditions, thus indicating that free radicals are produced in muscle and liver during resting metabolism prior to stimulation by exercise.

Vitamin E deficiency resulted in a lesser increase in ESR signal intensity in both muscle and liver of exercise-exhausted rats. The authors further suggest that the damage induced by exercise is gradual and cumulative.

Exhaustive exercise also led to a loss of sarcoplasmic reticulum and endoplasmic reticulum integrity together with an increase in lipid peroxidation products. Lipid peroxidation was measured using the thiobarbituric acid reactive substances (TBARS) method. Lipid peroxidation was greatly increased by both exercise and vitamin E deficiency (see table 2.0, overleaf).

Table two shows that there is a large variation in concentration of both free radicals and lipid peroxidation. Vitamin E-deficient rats exhibit higher baseline levels of lipid peroxidation in both muscle and liver homogenates.

Vitamin E is known to act as a chain breaking antioxidant and it is worth noting that elevated free radical concentrations observed to accompany exercise and vitamin E deficiency were always associated with increased lipid peroxidation. Elevated levels of conjugated dienes, indicative of increased lipid peroxidation, were seen in isolated mitochondria. Also reported was a decrease in mitochondrial respiratory control suggesting an inner mitochondrial membrane leakiness and decreased energy coupling efficiency.

Table 2.0: Free radicals and lipid peroxidation produced by exercise.

	Control Rats.				Vit. E Deficient Rats.			
	Muscle homogenate		Liver homogenate		Muscle homogenate		Liver homogenate	
	Rested	Exercised	Rested	Exercised	Rested	Exercised	Rested	Exercised
Radical concen-tration	8.0 ±0.7	17.0±2.4	8.3±0.4	19.5±3.4	10.6±0.4	13.7±1.7	11.9±0.8	14.7±1.1
Lipid peroxid-ation	27.7±6.9	50.1±1.0	32.9±6.0	77.3± 10.2	39.3±6.1	45.1±9.3	61.0±7.6	79.8± 11.6

Source: Davies et al (1982). Results expressed as mean ± SEM. Arbitrary units are used for ESR results, it is assumed $\mu mol.L^{-1}$ for lipid peroxidation although this is not stated.

Surprisingly however, the post-exercise increase in lipid peroxidation is greater in muscle homogenate of rats in the control group compared to those in the vitamin E deficient group. This is almost replicated in liver homogenate of non-deficient vs deficient rats, with a small post-exercise increase in the vitamin E-deficient rats of 77.3 vs 79.8 respectively. Logically it could have been expected that the vitamin E-deficient rats would have experienced a greater increase in lipid peroxidation of the active muscle tissue. This apparent discrepancy was explained by the fact that the vitamin E-deficient rats had lower endurance levels, and thus perhaps could not sustain exercise for a long enough duration to observe the remarkable increase seen in the baseline levels of non-deficient rats.

The ESR results exhibited higher concentrations of free radicals at baseline in both the muscle and liver homogenates of the vitamin E-deficient rats. Again, suprisingly perhaps, a greater increase was observed in the post-exercise muscle

homogenate of the non-deficient compared to the deficient rats, 17.0 vs. 13.7 respectively. This was replicated in the liver samples, the explanation may be that the lack of vitamin E prevented exercise duration continuing to where large increases in ESR signal intensity can be observed.

Jackson et al (1985).

Jackson et al (1985) examined the effect of 30 min^{-1} electrically-stimulated muscle contraction in the gastrocnemius of male Wistar rats (n = 6). The authors also examined the ESR spectra from mouse and human muscle. Excessive contractile activity resulted in a 70% increase in the amplitude of the ESR signal intensity. The ESR analysis was performed using a Varian E-109 or E-3 spectrometer at 77 K. Intact muscle samples as well as homogenates were used in this study and one major ESR signal was detected in all samples at 77 K. Room temperature analysis was also carried out but no signal was detected in either thawed intact muscle samples, or in liquid homogenates at room temperature.

Excessive contractile function also resulted in a leakage of intra-cellular creatine kinase into the blood plasma thus indicating exercise-induced damage to the muscle membrane. A g value of 2.0036-2.004 was ascribed to the signal by comparison to the stable free radical signal obtained from 1,1-diphenyl-β-picrylhydrazyl (DPPH). In addition to this, ESR signals characteristic of a nitroxide adduct were obtained by the use of muscle homogenates in a 25mM solution of the spin trap N-tert-butyl-α-phenylnitrone (PBN) and 2-methyl-2-nitrosopropane (MNP). Again these were detected at 77 K but were absent at room temperature. However a doublet of approximately 120 Gauss (12.0 mT) was seen in some samples at room temperature and was attributed to a phosphorus-centred radical.

The general absence of a signal at room temperature is indicative of the ability of water molecules to absorb microwaves and thus block the signal, which may be one explanation for the absence of spectra in the work by Davies et al (1982). It is also possible that the absolute concentration of radicals in the sample were very low. The authors state that it is their belief that the signal was present at room temperature since on thawing and re-freezing of the samples the signal re-appeared. They rightly stress that a loss of instrument sensitivity when using liquid aqueous samples is the reason for the absence of the signal at room temperature.

A large reduction in instrument sensitivity is known to occur when liquid aqueous samples are used since water possesses a high di-electric constant and a large damping loss for any oscillating magnetic field in which it is placed (Ingram 1969). The inability to detect a signal at room temperature would suggest that the concentration of radicals may be very low and beyond the limit of detection of the spectrometer, and also interference by the aqueous nature of the sample. The ESR signal obtained in the study compares well with that reported previously with the exception that no signal was detected in room temperature samples which is at variance with earlier work by Davies et al (1982).

The origin of the signal is unclear and may be semi-quinone although this would be difficult to confirm. Furthermore absence of narrow isotropic features in the spin trapped samples indicate, as stated by the authors, that only high molecular weight bio-polymer radicals were trapped. This could feasibly include chain carrying carbon centred lipid-derived radicals.

It is worth noting that in both the previously mentioned studies gastrocnemius muscle tissue was used. This is potentially significant in that the type of fibre that

predominate in this muscle are slow twitch oxidative fibres. It would be useful to determine the equivalent ESR spectra from fast twitch glycolytic fibres. Perhaps this would yield different ESR spectra and could therefore be used to make statements regarding the origin of the radicals. ESR evidence of this nature is currently lacking.

Human rectus abdominus samples were obtained and analysed by Jackson et al (1985) under the same ESR conditions, ie. at 77 K. A virtually identical signal was detected, thus indicating that the radicals present were not species-specific.

Kumar et al (1992).

Kumar et al (1992) used ESR to examine the effect of dietary vitamin E supplementation on exercise-induced oxidant stress in rat heart tissue. Female Wistar albino rats (n = 6), were forced to swim to exhaustion daily for a prolonged duration (60 days) before being sacrificed. Samples of myocardium and serum were analysed for evidence of exercise-induced free radical production using ESR spectroscopy and: MDA (TBARS) test; superoxide dismutase (SOD); xanthine oxidase (XO); catalase; and selenium-dependent (Se-GPX) and independent (non-Se GPX) glutathione to provide supporting data.

Room temperature ESR results were obtained with a JEOL JES FE3X spectrometer using the following operating conditions; microwave frequency 9.215 GHz, incident microwave power 20 mW, amplitude 3.2 x 10^3, modulation 6.3 gauss (0.63 mT), scan rate 4.0 min. time constant 0.30 sec. Concentration of radicals present were calculated from signal peak heights and were expressed in arbitrary units. The control and exercised rats exhibited an ESR signal that compares favourably to that previously reported (Davies et al 1982, Jackson et al 1985).

Furthermore the addition of 220IU/Kg body weight of vitamin E to the diet completely abolished the ESR signal in the heart homogenate of the exercise exhausted rats. This is in direct comparison to non-supplemented rats which exhibited a signal (g = 2.074), similar to that previously demonstrated by Davies et al (1982). In control animals daily exhaustive exercise resulted in significant increases in tissue MDA compared to vitamin E-fed animals.

Tissue levels of SOD were elevated in response to exercise in both control and exercised rats. Exercise also decreased Se-GPX significantly in control exercised animals whereas no such decrease was observed in vit E supplemented exercised animals. However, non-Se GPX increased significantly in both vitamin E-supplemented and non-supplemented exercised animals. Xanthine oxidase activity also increased in both control and vit E supplemented animals although the levels of the vitamin E-supplemented animals were below the non-supplemented levels.

Tissue MDA was increased in the heart of both non-supplemented and supplemented exercised groups although the level of tissue MDA was lower in the vitamin E supplemented group. The authors concluded that exhaustive endurance exercise in female albino rats resulted in free radical mediated oxidant stress and that this was attenuated by dietary vitamin E.

Borzone et al (1994).

The earlier work of Jackson et al (1985), was confirmed by a study examining the effect of resistive loading on rat diaphragm, results demonstrated the existance of a virtually identical ESR signal to that previously published.

The above studies, with the exception of Jackson et al (1985), generally used ESR spectroscopy without the spin trap technique. Evidence will now be presented from workers using ESR in conjunction with spin traps.

Sommani and Arroyo (1995).

Sommani and Arroyo (1995) demonstrated the existence of a triplet of doublets characteristic of the PBN adduct in the heart tissue of exercise-trained rats concluding that exercise causes free radical production. The hyperfine coupling constants of the spin adduct were; a_N = 1.63 mT, and a_H = 0.35 mT.

Furthermore the authors detected the presence of the ascorbyl radical (a_H = 0.189 mT) which was not evident in the control. This appears to be the only study, apart from those reviewed earlier, that specifically used the spin trap PBN in the study of exercise-induced oxidative damage / stress.

It can be seen from the above mentioned studies that strenuous aerobic exercise causes an increase in free radical concentration, and that ESR is a useful and applicable technique for the detection of this increase in free radicals both in spin trapped as well as tissue samples. Furthermore that it is applicable to the study of exercise-induced free radical production in animals and should therefore be applicable humans.

Table 2.1 shown overleaf, summarises the published ESR work relating to exercise-induced free radical production.

Table 2.1: Summary of ESR data and free radical production.

Author	Model	Stress	Results
Davies et al (1982)	Rats - skeletal muscle tissue	Exhaustive exercise	Increase in ESR signal
Jackson et al (1985)	Rats - skeletal muscle tissue	Electrical stimulation	Increase in ESR signal
Kumar et al (1992)	Rats- heart tissue	Exhaustive exercise	Increase in ESR signal
Borzone et al (1994)	Rat- diaphragm	Resistive loading	Increase in ESR signal
Sommani and Arroyo (1995)	Rats- heart tissue	Exercise	Increase in ESR signal (PBN adduct and ascorbyl radical)

It can be seen from the above table that there is strong agreement between the different groups, each demonstrating post-exercise increases in the ESR signal intensity. It is fair to say that the paucity of human ESR experimental data represents a serious oversight within the scientific community ! Currently, to this authors knowledge, there are no published studies utilising the technique of ESR with or without spin trapping using healthy human subjects and an exercise model.

2.3.2: LIPID PEROXIDATION PRODUCED BY EXERCISE.

Lipid peroxidation is arguably the most widely studied aspect of exercise-induced oxidative stress. A reason for this may be that the peroxidation of

membrane lipids is a recognised phenomenon following free radical attack. Additionally, in an attempt to prevent lipid peroxidation vitamin E appears to have been a frequently utilised antioxidant intervention. This is perhaps due to its lipid solubility and action in the lipid bi-layer of cell membranes.

First chain initiation of membrane lipid peroxidation may well involve hydrogen atom abstraction by the hydroxyl radical from an unsaturated lipid methylene (-CH_2) group, forming a carbon-centred radical. This results in propagation of peroxidation and the formation of other secondary lipid- derived free radicals, such as peroxyl or alkoxyl radicals. The process will terminate when the chain reaction is halted by a low molecular weight chain- breaking antioxidant, such as vitamin E, or one radical colliding with another whereupon the unpaired electrons pair off and stabilise the molecule. Alternatively ascorbic acid may also scavenge aqueous peroxyl radicals and lead to termination.

Several products are produced as a result of membrane lipid peroxidation including the aldehydes MDA and 4-hydroxynonenal, conjugated dienes and lipid hydroperoxides. In the order of production of lipid peroxidation products conjugated dienes are thought to appear first followed by lipid hydroperoxides and malondialdehyde (Kneepkens 1994).

The volatile hydrocarbons ethane and pentane have also been studied in the breath of exercising subjects. It is pertinent to point out that many studies have examined the effects of exercise on levels of lipid peroxidation in both animal and human subjects. In so doing these studies have often measured two or more indices, for example MDA and lipid hydroperoxides. Therefore a large amount of overlap is to be expected when attempting to review the literature. Also there are different techniques in use for example the thiobarbituric acid reactive substances (TBARS) test and HPLC assays for MDA.

It is worth noting that the variety of available techniques might suggest a lack of specificity or sensitivity. This is particularly true of the TBARS method. This combined with differing exercise protocols cited in the literature have contributed to the variable results reported. Where possible human studies have been reviewed in preference to animal studies.

CONJUGATED DIENES.

Diene conjugates tend to be rapidly formed once peroxidation of membrane lipids has been initiated (Pryor and Castle 1984). In the presence of oxygen conjugated dienes are able to form lipid peroxyl radicals and lipid hydroperoxides. Conjugated dienes are polyunsaturated molecules having two double bonds separated by a single bond. Oxygen-free radicals are able to attack this molecule resulting in one or more of the double bonds shifting to become diene conjugated.

It has been reported that there is no significant difference in the plasma concentration of diene conjugates between male high and low training runners and sedentary controls (Roberston et al 1991). The same group had earlier reported no significant change in conjugated dienes following a half marathon (Duthie et al 1990).

The level of conjugated dienes studied during exercise has not been restricted to plasma. Levels of conjugated dienes in synovial fluid have been examined, and no significant difference was found between isometric quadriceps contraction or level walking (Merry et al 1991). Thus there appears to be consistency in the results of several workers regarding the influence of exercise on the level of conjugated dienes found not only in blood plasma but also synovial fluid. The determination of conjugated dienes in human body fluids is fraught with

difficulty (Banni et al 1990, Gutteridge and Halliwell 1990). Coupled with the lack of change observed in several studies its usefulness as a marker of *in vivo* lipid peroxidation in exercise-induced oxidative stress must surely be questioned.

LIPID HYDROPEROXIDES (LH).

LH are the major initial reaction products of lipid peroxidation, this therefore makes them particularly useful as a marker of free radical-mediated lipid peroxidation (Pryor and Castle 1984).

In relation to exercise relatively few studies have been performed examining LH levels either pre, during or post-exercise, although muscle LH has been shown to increase by 33% following high intensity exercise (Alessio et al 1988). However a recent report failed to detect LH in plasma following 90 min. of cycle ergometry at 65% of maximal oxygen uptake (Viguie et al 1993). This moderate exercise protocol however, did induce an oxidative stress as evidenced by increased rates of plasma glutathione (GSH) oxidation. It is feasible that the moderate nature of the exercise, although causing oxidation of GSH and thus an oxidative challenge, was insufficient to cause lipid peroxidation, indicating that the antioxidant defence mechanisms were able to cope with this low level of exercise-induced oxidative stress.

Moderate exercise did not result in significant increases in LH in several studies reported by different groups (Ji et al 1988, Salminen and Vihko 1983, Viinikka et al 1984). However a 96% increase in blood LH above pre-exercise levels following exhaustive exercise has been reported (Alessio and Cutler 1990).

Reported measurements of plasma lipid hydroperoxides reveal increased levels in patients suffering from oxidative-stress related disease states, notably diabetes mellitus and cystic fibrosis but also patients undergoing coronary angioplasty (Brown and Kelly 1996). This clearly suggests that oxidative stress induces measurable increases in plasma lipid hydroperoxides.

The implication therefore from an exercise perspective is that the exercise must be sufficiently strenuous in order to induce measurable increases in the levels of lipid hydroperoxides found in plasma irrespective of training state. This is further supported by the fact that changes in plasma lipid peroxides are more dependent upon the extent or intensity of acute physical exercise rather than on previous training state (Kretzschmar et al 1991).

MALONDIALDEHYDE (MDA).

MDA is arguably the most widely studied aspect of exercise-induced lipid peroxidation having been assessed in both animal and human models. Of the studies examining the influence of exercise on MDA it is fair to say that the majority measure plasma or serum levels using the thiobarbituric acid reactive substances (TBARS) assay. Results of the various studies have however been equivocal. This again, may well be due to the variation in methods and exercise protocols used in the plethora of studies, which unfortunately prevents accurate comparison between studies. However it is again possible to identify a trend to increased MDA following strenuous exercise in both animals and humans.

A pertinent point to note relates to the results obtained when comparing trained vs. untrained subjects. Generally trained subjects do not show as large an increase in tissue MDA thus indicating a protective effect of training which is evident in animal and human subjects (Salminen and Vihko 1983, Viinikka et al

1984, Alessio and Goldfarb 1988). The suggested mechanism of protection involves increased activity of the antioxidant enzymes such as superoxide dismutase as demonstrated by Jenkins et al (1984).

The effect of exercise eliciting maximal oxygen uptake on blood MDA concentration has been studied in six (n = 6) male physical education students (Lovlin et al 1987). The subjects were required to cycle to voluntary (volitional) exhaustion on a calibrated Monark cycle ergometer. Oxygen uptake and heart rate were recorded every 30 s. using a Beckman metabolic cart. The exercise protocol included a 5 min^{-1} warm up and resistance was increased by 30W every minute until exhaustion. Results indicate a 26% increase ($p < 0.005$) in plasma MDA from 2.26 $mmol.L^{-1}$ at rest to 2.88 $mmol.L^{-1}$ at exhaustion. They also report a significant decrease in plasma MDA at 40% VO_{2max} and that at 70% VO_{2max} MDA was still below resting values. Significantly though they describe a definite trend to increased MDA with increasing exercise intensity.

Kanter et al (1993) reported a 20% increase in serum MDA in both trained and untrained male runners following a treadmill test at 60% and 90% of VO_{2max}. In an earlier study, the effects of an eighty kilometre race on the serum MDA levels of nine male runners pre and post-exercise were examined. The authors reported an almost 100% increase in serum MDA post vs. pre-exercise (Kanter et al 1988). They also report a correlation between MDA and creatine kinase thus implicating exercise-induced muscle damage in increased amounts of lipid peroxidation products.

In another study, Sen et al (1994) examined the effect of n-acetyl-cysteine supplementation in nine (n = 9) male subjects. The authors reported a 50% and 100% increase in plasma MDA following sub-maximal cycle ergometry at the corresponding aerobic and anaerobic threshold respectively (Sen et al 1994).

Interestingly they failed to notice any change in plasma MDA following a graded 14min^{-1} cycle ergometer test. It is possible that the exercise was of insufficient duration and/or intensity to induce measurable increases in MDA.

Urinary excretion of MDA in young and old men following 15 min^{-1} of downhill running (gradient = -16%), on a treadmill corresponding to 75% of maximal heart rate has been reported (Meydani et al 1993). Results indicated a greater than 60% increase in urinary MDA on the twelfth day post-exercise but not on the same day or on the other days examined which were: 1, 2 and 5 days post-exercise. Results were compared to the day before the exercise test. However, the authors also noted a greater than 100% increase in vastus lateralis MDA content immediately post-exercise and a greater than 175% increase in samples collected 5 days post-exercise (Meydani et al 1993). The increase in muscle MDA importantly implicates active skeletal muscle as a source of lipid peroxidation products during aerobic exercise.

It is feasible therefore in view of these findings that skeletal muscle mitochondrial electron transport chain leakage, and consequent free radical formation is the origin of the observed increase in MDA, which may diffuse into and thus be measured in blood. This also suggests a time course of production and clearance of products of oxidative damage. In support of this MDA levels have been shown to peak six hours after a 45 min. downhill treadmill run, while levels of creatine kinase were also significantly elevated (Maughan et al 1989).

In addition to using ESR spectroscopy a greater than 80% and 100% increase in muscle and liver MDA respectively, following a sub-maximal treadmill run to exhaustion was reported (Davies et al 1982). Similarly a significant increase in plasma MDA following a sub-maximal treadmill run to exhaustion in male Han-Wistar rats has also been reported (Sen et al 1994). This supports the earlier work

of Jenkins and Goldfarb (1993) who noted an approximately 70% increase in urinary MDA of trained and untrained rats.

The study of exercise-induced lipid peroxidation has not been confined to humans and rodents, or to plasma and tissue samples. The effect of selenium supplementation on the response of the equine erythrocyte glutathione system to exercise-induced oxidative stress has been studied (Brady et al 1978). The authors noted an almost 100% increase in MDA content of venous erythrocytes immediately after exercise (Brady et al 1978).

Therefore it would appear that increases in products of free radical-mediated lipid peroxidation occur in a number of tissues, irrespective of species. The common denominator throughout the studies is the large increase in whole body oxygen uptake induced by exercise.

As stated previously results from the literature are equivocal, a randomized, double-blind, placebo controlled study examining the effect of what was described as extreme endurance stress on trained athletes was performed by Rokitzki et al (1994). Twenty two (n = 22) well-trained male athletes participated in the study. The exercise involved a marathon at 1,000 metres of altitude in relatively cold temperatures (9-12 °C). In addition to measuring MDA, athletes were supplemented with 400 $IU.d^{-1}$ α-tocopherol and 200 $mg.d^{-1}$ ascorbic acid or a placebo. (The results of the antioxidant supplementation will be discussed in the section on antioxidants and exercise, see section 2.3). Results show a decrease in MDA measured immediately after the race in both the supplemented and placebo group (Rokitzki et al 1994). It is important to note that the subjects were well-trained athletes since one of the adaptations to training may be an enhancement of the antioxidant defence mechanisms (Criswell et al 1993, Dernbach et al 1993).

Workers have concentrated on endurance exercise when attempting to measure exercise-induced free radical production and consequent lipid peroxidation. This therefore implicates aerobic metabolism as a source of free radical generation. The possibility certainly exists that mitochondrial electron transport chain leakage is the origin of this exercise-induced oxidative stress.

In support of the hypothesis in this thesis, repetitive static muscle contraction was shown to have no effect on the plasma MDA content of four (n = 4) male volunteers (Sahlin et al 1992). This supports the hypothesis, since in repetitive static muscle contraction whole body oxygen uptake is not raised significantly.

The energy for muscular work would be provided primarily by creatine phosphate and anaerobic glycolysis, a small sample number (n=4) however, is a relevant criticism of the above mentioned study. The authors also reported no effect of dynamic exercise at 60% of maximal oxygen uptake, which is in agreement with previously published data (Lovlin et al 1987). This again suggests that aerobic exercise needs to be sufficiently strenuous to induce measurable increases in products of lipid peroxidation, and furthermore that a threshold of exercise-induced oxidative damage/stress exists.

It can be seen therefore from the numerous studies that in general strenuous aerobic or endurance exercise, eliciting large increases in whole body oxygen uptake, causes measurable increases in the products of lipid peroxidation following attack by oxidants on such things as cell membrane PUFA.

PENTANE PRODUCTION BY EXERCISE.

Pentane is a minor decomposition product of n-6 polyunsaturated fatty acids arachidonic and linoleic acid. It is produced via β-scission of lipid alkoxyl

radicals (Kneepkens et al 1994). The earliest report of an exercise-induced increase in lipid peroxidation was published by Dillard et al (1978). The authors reported a 1.8-fold increase in pentane excretion among six (n = 6), male and female student volunteers following exercise at 75% VO_{2max} for 20 min. A spirometer was used to eliminate high background pentane levels which was found to be 18-40 pmol/100ml room air. They suggest that the origin of the increase in pentane excretion are the microsomes of various organs such as the liver. This also implicates movement of pentane from the liver, presumably via the systemic circulation, to the lungs where it is exhaled. This illustrates the possibility that products of free radical-mediated lipid peroxidation may diffuse away from their site of origin to cause damage and be measured at other sites.

One interesting finding of Dillard's study is that the administration of 1200 IU dl-α-tocopherol/day for two weeks resulted in a significant reduction in expired pentane levels. This work has been subsequently confirmed and a 310% increase in breath pentane, and activation of neutrophils following a graded cycle ergometer test has been reported by Pincemail et al (1990).

It has been suggested that peroxidation of polyunsaturated fatty acids, known to occur as a result of strenuous exercise, is the major endogenous source of ethane and pentane found in the breath (Kneepkens et al 1994). Recently however analysis of pentane as an index of lipid peroxidation has been criticised (Gutteridge and Halliwell 1990).

2.4 ANTIOXIDANTS AND FREE RADICAL PRODUCTION: AN INTRODUCTION.

Over the past decade much attention has been paid to the role antioxidants play in physical activity and performance (Demopoulos et al 1986). Interest has appeared to have extended to the potential therapeutic effects of antioxidants (Gilligan et al 1994), their role in the prevention of disease (Diplock 1993, Polli and Parola 1997, Steinberg 1991, Gey 1990), and even the slowing down of the aging process (Cutler 1991, Ames 1989).

Unfortunately much of the information presented in the context of health and exercise appears not to be based on strong scientific research, but on anecdotal evidence and misinterpreted research findings. Advertisements in many sport and health magazines advocate antioxidant supplementation for anything from delaying aging to improving sports performance. The attention has largely been focused on the effects antioxidants may have on the increased amounts of oxygen-free radicals produced as a consequence of strenuous aerobic exercise, and particularly, their use as ergogenic aids to enhance performance. In addition to this, since many pathologies have free radicals implicated in their aetiology and progression, their use as therapeutic agents has become increasingly widespread.

Epidemiological evidence has implicated poor antioxidant intake in the development of cardiovascular disease (Stampfer et al 1993, Rimm et al 1993). It must be stressed however that epidemiological data merely show associations, not cause and effect. To this end the Cambridge Heart Antioxidant Study (CHAOS), a randomised controlled trial of the effectiveness of vitamin E supplementation in patients with coronary disease was carried out. Results demonstrated a significant reduction in non-fatal myocardial infarction after one year of treatment with 400 or 800 IU α-tocopherol (Stephens 1996). These data

demonstrate a role for free radical mediated pathology in cardiovascular disease probably via the atherogenic potential of oxidized LDL.

Thus any decline in dietary intake of antioxidant vitamins coupled with unaccustomed or strenuous physical activity would mean that the possibility of exercise-induced oxidative stress and concomitant tissue damage is amplified. This would be particularly relevant to those people who exercise sporadically and may require supplemental antioxidants, because their antioxidant defence mechanisms may be unable to deal with a sudden influx of oxygen-centred radicals. This inability to deal with excess free radical production is further compromised by a lack of enhancement of antioxidant defence mechanisms brought about by regular physical training. Thus the link between exercise-induced oxidative stress and antioxidant supplementation needs to be explored.

One of the aims of this research is to determine the effect of antioxidant supplementation on exercise-induced oxidative stress.

2.4.1 Definition and Mechanism of Action of an Antioxidant.

An antioxidant may simply be defined as a compound, usually organic, that prevents or retards oxidation by molecular oxygen and thus may confer some protection from the damaging effects caused by free radicals (Kent 1994). An even broader definition is below:

> "an antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate significantly delays or inhibits oxidation of that substrate"
>
> (Halliwell and Gutteridge 1989).

The authors further describe the possible mechanisms by which antioxidants may act. For example, antioxidants may act by preventing first chain initiation - the abstraction of a hydrogen atom from a cell lipid bi-layer - by scavenging initiating radicals such as $^{\cdot}OH$ or $O_2^{\cdot-}$. They may act by binding transition metal ions, notably iron and copper to transferrin and caeruloplasmin, respectively, preventing them from catalysing the formation of initiating species and decomposing lipid peroxides to aqueous peroxyl or alkoxyl radicals (Halliwell and Gutteridge 1989). They may also act by scavenging intermediate radicals such as peroxyl and alkoxyl radicals thus preventing them from continuing to abstract hydrogen, vitamin C is thought to work in this manner. They may also act as chain breaking antioxidants, such as vitamin E.

The human body is relatively well endowed with antioxidant defences of both an enzymatic and non-enzymatic nature. The most abundant enzymaticic antioxidants include glutathione peroxidase and catalase, whose chief role is to decompose hydrogen peroxide to water and molecular oxygen, and superoxide dismutase (SOD) whose role is to specifically catalyse the dismutation of superoxide to hydrogen peroxide and molecular oxygen. The superoxide theory of oxygen toxicity illustrates the need to remove this damaging free radical (McCord and Fridovich 1969).

Superoxide dismutase has several metallo-cofactors notably, copper and zinc (CuZn-SOD - which mainly resides in the cytosol of the cell), and manganese (Mn-SOD - which is mainly concentrated in the mitochondrial matrix) (Halliwell and Gutteridge 1989). Superoxide anion is a rather unstable species and a common intermediate of oxygen reduction which is a consequence of the fact that ground state oxygen prefers univalent pathways of reduction (Fridovich 1975).

There is a linear relationship between both superoxide dismutase and catalase activity and maximal oxygen uptake and subjects with a high aerobic capacity have been reported to have greater levels of these enzymes compared to subjects with a lower VO_{2max} (Jenkins et al 1984).

It is of note that the human body does not appear to contain any enzymatic means for the specific removal of the hydroxyl radical. One interpretation of this is that the body is not designed to produce the hydroxyl radical. Therefore if this highly reactive free radical is produced the chief defence mechanism relies on the antioxidant nutrients such as vitamins C and E.

Antioxidants may be located either intra or extracellularly. It is fair to say that the water soluble antioxidants primarily tend to be located extracellularly whilst the lipid soluble antioxidants are mainly confined to the lipid bi-layer of the cell membrane. An example of each of these types of non-enzymatic antioxidants is vitamin C and vitamin E respectively.

2.4.2 Ascorbic acid biochemistry.

Vitamin C is the generic name for substances that provide the biological activity of L-ascorbic acid ($C_6H_6O_8$). Ascorbic acid is a ketolactone (MW 176.1), whose molecular structure contains two enolic hydrogen atoms which are readily ionizable yielding its acidic character. The enediol group and furane ring structure are essential for vitamin C activity, with the most biologically-active substances being L-ascorbic acid and L-dehydro-ascorbic acid (Elmadfa and Koenig 1996). Figure 2.7 shows the antioxidant properties of ascorbic acid.

FIGURE 2.7: THE ANTIOXIDANT PROPERTIES OF ASCORBIC ACID.

ASCORBATE ⇔ ASCORBATE FREE RADICAL ⇔ DEHYDROASCORBATE ⇒ OXALIC ACID

⇓

L-THREONIC ACID

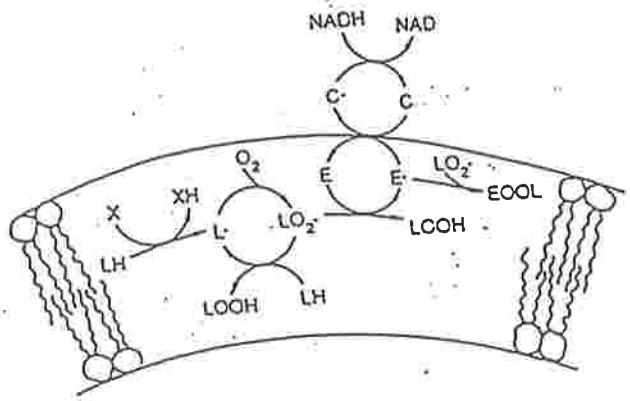
Since ascorbic acid has been selected as the antioxidant intervention in this research, there follows a review of studies that have used ascorbic acid as a supplement during exercise to attempt to influence performance and/or modify exercise-induced free radical production. Also a small selection of pertinent studies regarding the health-related aspects of ascorbic acid are reviewed.

Ascorbic acid may well play a central role in the armour of defence against free radical attack via its antioxidant role as a scavenger of aqueous chain carrying peroxyl radicals and its ability to regenerate α-tocopherol from the tocopheroxyl radical (Bendich et al 1986). Direct evidence using ESR of the synergistic relationship between vitamins C and E has been reported (Packer et al 1979), and vitamin C has been shown to regenerate the tocopheroxyl radical in biological systems with a second order rate constant of 322 $M.s^{-1}$ at pH 7 (Mukai et al 1991).

A noteworthy point is that vitamin C can react directly with aqueous chain carrying peroxyl radicals which may degrade to alkoxyl radicals (Bendich et al 1986). Furthermore ascorbic acid is the only low molecular weight antioxidant that is able to actively scavenge aqueous peroxyl radicals and alkoxyl radicals. It is also preferentially oxidized in relation to vitamin E (Dr. Balz Frei - personal communication). The potential for ascorbic acid to scavenge aqueous peroxyl/alkoxyl radicals supports its use as an antioxidant intervention. Ascorbate has also been shown to reduce spinal cord injury in rats by scavenging aqueous peroxyl radicals (Katoh et al 1996) and to improve endothelial

dysfunction in smokers (Heitzer et al 1996). Figure 2.8 below illustrates the synergistic relationship between vitamins E and C in the prevention of membrane oxidation.

Figure 2.8: Synergistic inhibition of oxidation of membranes by vitamin C and E.



Where: LH is unsaturated fatty acid, L· is allyl radical, LO_2· is peroxyl radical, LOOH is lipid hydroperoxide.

(Niki 1991).

Interestingly the administration of 2g per day of vitamin C for 5 days was shown to suppress urinary levels of 8-epiprostaglandin F2 alpha a product of *in vivo* lipid peroxidation derived from arachidonic acid (Reilly et al 1996). Significantly this effect was noted with vitamin C alone, or in combination with vitamin E, but not in vitamin E alone, thus suggesting that ascorbic acid is able to modulate *in vivo* lipid peroxidation despite being a water soluble antioxidant. This is again important since in this thesis there is an exercise and antioxidant intervention study and an *in vitro* study using arachidonic acid and ascorbic acid.

Numerous studies have examined the antioxidant role of vitamin C in areas as diverse as cancer prevention (Block and Menkes 1989) and enhancement of immune function (Anderson 1984).

A recent paper suggests that dietary intake of vitamin C is low in UK males and hence plasma ascorbate is low compared with females, it is further suggested that resistance to the common cold is also reduced (Hemila 1997). It has been recently suggested that vitamin C deficiency as assessed by low plasma concentrations (<11.4 $\mu mol.L^{-1}$) is associated with an increased risk of coronary heart disease (Nyyssonen et al 1997).

2.4.3 ANTIOXIDANTS AND EXERCISE.

TOTAL ANTIOXIDANT CAPACITY DURING EXERCISE.

Few studies have measured plasma total antioxidant capacity (TAC), in relation to exercise-induced oxidative stress. In a randomised controlled study the effect of one hour of box-stepping exercise on a range of free radical markers was examined by Maxwell et al (1993). Twenty four (n = 24), male and female students were randomly divided into three groups who received either: no supplementation; or 400 mg/day of ascorbic acid; or dl-α-tocopherol acetate, for two weeks prior to the test. Supplementation was continued for one week after the test. Results indicate a significant elevation in plasma TAC as measured by enhanced chemiluminescence (Whitehead et al 1992), following one hour of box stepping exercise. Following supplementation, perhaps as expected, plasma levels of ascorbic acid and vitamin E were higher in the supplemented compared to the unsupplemented group. Although there was no significant increase in basal TAC. This may be explained by the fact that vitamins C and E are thought

to account for less than 50% of the plasma total antioxidant capacity (Whitehead et al 1992, Wayner et al 1987).

In Maxwell et al's (1993) study, the post-exercise increase in plasma TAC was more significant in the supplemented groups vs. un-supplemented group ($p < 0.05$, $p < 0.001$) respectively. Also a significant post-exercise increase in ascorbic acid was observed in the supplemented group possibly reflecting increased availability or mobilisation from tissues. A point of note is that there was no significant increase in plasma MDA post-exercise. This suggests either the exercise was not strenuous enough, that the MDA remained in the muscle and did not migrate to the blood stream, or that the increased level of plasma antioxidants quenched any oxidants produced.

This is a paradox because box stepping may be described as a primarily eccentric exercise since the muscle fibres are lengthened as the muscle develops force. Eccentric exercise is known to induce muscle damage resulting in release of enzymes and disruption of contractile properties of skeletal muscle (Newham et al 1987). It could therefore be expected that this type of exercise would certainly induce measurable increases in products of oxidant-mediated attack, if not due to the metabolic stress of the exercise itself then because of muscle damage. One explanation for this may be that oxygen consumption by skeletal muscle is thought to be less during eccentric exercise compared with concentric exercise (Jackson 1994).

It is likely that where tissue damage occurs then lipid peroxidation is an inevitable consequence. It is therefore tempting to speculate that the antioxidant supplements protected the tissues against free radical-mediated oxidative damage. This conclusion is further supported by the significant rise in creatine kinase post-exercise reported in the study, which suggests that creatine kinase is

not readily amenable to modulation by antioxidants. However, the fact that creatine kinase is increased suggests exercise-induced muscle damage (Clarkson and Ebbeling 1988). There is a strong correlation (r = 0.85) between post-exercise MDA and muscle-derived creatine kinase (Kanter et al 1988). Thus any tissue damage is likely to result in an increase in MDA.

One conclusion from the data presented by the box stepping study is that total antioxidant capacity of the plasma rises post-exercise and that supplementation with vitamins C and E may well prevent a post-exercise increase in products of free radical-mediated lipid peroxidation including MDA.

VITAMIN E AND EXERCISE.

Although in this thesis the effect of ascorbic acid as an intervention in exercise-induced free radical production is examined, a review of the literature indicates that the majority of published work appears to concentrate on vitamin E as an intervention. One possible explanation for this is that many studies have examined the phenomenon of exercise-induced lipid peroxidation, and since vitamin E is lipid soluble then workers have tended to concentrate on vitamin E. A modest selection of the most relevant studies are reviewed with particular emphasis on those that use vitamins E and C in combination.

Results of studies using vitamin E in isolation or in combination with other biological antioxidants have been equivocal in both human and animal models. The effect of elevated muscle vitamin E content on skeletal muscle damage induced by eccentric exercise has been studied by Warren et al (1992). Sixty (n=60) female Sprague-Dawley rats were assigned a normal (40 IU /vitamin E/Kg/day) or a supplemented (10,000 IU /vitamin E/Kg/day) diet for 5 weeks. Muscle damage was induced by a total of 150 min^{-1} of walking on a downhill (-

17°) motor-driven treadmill. Vitamin E supplementation resulted in greater levels of muscle vitamin E content of approximately 3 to 4 fold vs. un-supplemented animals and susceptibility to oxidative stress as measured by TBARS was decreased. However, vitamin E supplementation did not attenuate injury to soleus muscle using the criteria employed, ie. elevated plasma creatine kinase, or reductions in maximal tetanic force and number of intact fibres per square millimeter. The authors suggest therefore that free radicals do not play a critical role in damage to muscle membranes, although they point out that their evidence does not exclude free radical involvement in the aetiology of muscle damage.

The results of Warren et al (1992) are at variance with those of Goldfarb's group who demonstrated that vitamin E can attenuate exercise-induced oxidative stress in rat heart muscle (Goldfarb et al 1996). Significantly though, it must be pointed out that the study involved rats running uphill where the nature of muscle contractions would be a mixture of concentric and eccentric as opposed to predominanatly eccentric contractions during downhill running, and focused on heart rather than skeletal muscle tissue.

It was discussed earlier that creatine kinase is not responsive to antioxidant supplementation. However, the protective effect of vitamin E on exercise-induced oxidative stress was examined in a group of young and older adults (Meydani et al 1993). Following vitamin E supplementation (800IU dl-α-tocopherol per day) for 48 days, nine young (n = 9) and twelve older (n = 12) male volunteers performed 45 min. of eccentric exercise at 75% of VO_{2max} on a downhill treadmill. Supplemented subjects excreted less urinary TBARS twelve days post-exercise compared to unsupplemented individuals ($p < 0.05$). Unsupplemented subjects also had higher levels of muscle conjugated dienes following biopsy thus suggesting that free radicals may be involved, and

furthermore that vitamin E confers some protection against eccentric exercise-induced oxidative injury.

One of the reasons why results are equivocal may be the variation in exercise protocols and training status of the subjects used. It has also been suggested that vitamin E supplementation may benefit only those individuals who are initially vitamin E deficient (Kagan et al 1994).

ASCORBIC ACID AND EXERCISE.

Few studies have examined the role of ascorbic acid supplementation in relation to exercise-induced free radical production. Where vitamin C has been used as an intervention workers have tended to concentrate on its perceived ergogenic properties in relation to athletic performance. Performance studies will be reviewed followed by antioxidant intervention studies.

ERGOGENIC PROPERTIES OF ASCORBIC ACID.

Several studies have examined ascorbic acid as an "ergogenic aid" and again the results have been equivocal. In a randomised placebo controlled trial the effect of vitamin C on endurance performance during a standardised 12 minute exercise test (The Cooper Run) was examined (Gey et al 1970). Two hundred and eighty six males (n = 286) were randomly assigned either 1 gram of ascorbic acid daily or placebo. Following twelve weeks of training the subjects repeated the exercise test. Results indicated a negligible effect by vitamin C on endurance performance. Interestingly the authors did not modify the subjects' diet. This is unlikely to have any bearing on results since the oral administration of 1 gram of vitamin C will significantly raise plasma ascorbic acid levels (Jones 1983).

The effect of vitamin C administration on energy supply to the working muscle was studied in a randomised placebo controlled trial (Howald et al 1975). A cycle ergometer test to exhaustion (which was virtually identical to the cycle ergometer VO_{2max} test used in this research), was used to determine the ergogenic effect of vitamin C in thirteen (n = 13) males. Importantly when the results were examined in relation to physical working capacity at a given heart rate a significant improvement in performance was observed. A lower heart rate at a given submaximal workload is indicative of an improved work capacity/efficiency, thus supporting the ergogenic potential of vitamin C. The authors also observed a decrease in blood glucose and an increase in plasma free fatty acid concentration. The decrease in blood glucose may be due to a vitamin C induced increase in catecholamine secretion, and thus an adrenaline-induced decrease in liver blood flow leading to a drop in blood glucose. The higher free fatty acid concentration found in the plasma being due to accelerated lipolysis in adipose tissue mediated by catecholamine secretion. In summary of this paper, results suggest a more important utilization of free fatty acids as an energy source in working skeletal muscle, this glycogen sparing effect both in muscle cells and the liver suggests vitamin C has a beneficial effect on exercise performance.

The effect of vitamin E deficiency and vitamin C supplementation on exercise performance and mitochondrial oxidation in rats was examined by Gohil et al (1986). The results indicated increased tissue concentrations of vitamin C in the vitamin C supplemented rats ($p<0.05$). A 33% reduction in endurance capacity of vitamin E deficient vitamin C supplemented rats compared to control rats, but a 38% reduction in vitamin E deficient non vitamin C supplemented rats was noted. In support of the earlier observations of increased free fatty acid utilization reported by Howald et al (1975), a 99% increase in activity of hepatic palmitoyl carnitine-malate cytochrome c reductase in vitamin E deficient,

vitamin C supplemented rats at exhaustion was observed in this study. This is suggestive of an increase in β-oxidation of free fatty acids and therefore oxidative metabolism. This is consistent with raised oxygen uptake and hence increased leakage of electrons from the mitochondrial electron transport chain, thus implicating oxidative metabolism in free radical production. The authors concluded however that vitamin C supplementation at 3g/Kg diet does not counteract the detrimental effects vitamin E deficiency has on endurance performance.

ASCORBIC ACID, EXERCISE-INDUCED TISSUE DAMAGE AND OXIDATIVE STRESS.

In a randomised double-blind placebo controlled study twenty four (n=24) physically active male and female physical education students were given antioxidant vitamin supplements in order to assess the effects on eccentric exercise- induced muscle damage (Jakeman and Maxwell 1993). Subjects were given either placebo (n = 8), 400mg vitamin E (n = 8), or 400mg vitamin C (n = 8) orally for 21 days prior to and 7 days post-exercise. The eccentric exercise test consisted of 1 hour of box stepping. Following eccentric exercise maximum voluntary contraction (MVC) decreased to 75% of pre-exercise values ($p< 0.05$). No significant difference in MVC was observed immediately post-eccentric exercise compared to placebo group, while in the 24 hours post-exercise recovery of MVC was greater in the vitamin C supplemented group. The authors also examined the 20/50 Hz ratio of tetanic tension and found a significantly lower decrease post-exercise and in the initial phase of recovery in subjects supplemented with vitamin C, but not vitamin E. Also observed was a 13% increase in plasma total antioxidant capacity post-exercise representing a net efflux of antioxidants into plasma. The authors further conclude that prior vitamin C supplementation may exert a protective effect against eccentric

exercise-induced muscle damage. This is important since eccentric exercise-induced muscle damage is known to be mediated by free radical attack in humans (Packer and Viguie 1989).

Vitamin E has been shown to reduce exercise-induced myocardial oxidative stress (Goldfarb et al 1996), and urinary TBARS excretion (Meydani et al 1993). This is potentially significant since vitamin E is the chief lipid-soluble, chain-breaking antioxidant in blood (Burton et al 1983), and is regenerated by ascorbic acid via donation of an electron at the lipophobic chromanol head (Niki 1991). Thus illustrating an example of the indirect antioxidant properties of vitamin C.

Other groups have also demonstrated the ability of vitamin C to attenuate exercise-induced lipid peroxidation. Rokitzki et al (1994) reported a decrease in serum TBARS following oral administration of 200mg d^{-1} vitamin C and 400 IUd^{-1} α-tocopherol. This supports the observation of an attenuation by vitamin C but not vitamin E, of *in vivo* oxidative stress induced by cigarette smoking described by Reilly et al (1996). One interpretation of this is that exercise-induced oxidative stress and smoking-induced oxidative stress are synonymous.

It is worth stating however that there appears to be a substantial synergism between the antioxidant actions of vitamins C and E with ascorbic acid being preferentially oxidized. It may be that ascorbic acid is the sacrificial plasma antioxidant. This synergistic relationship is not confined to antioxidant vitamins but also involves the enzymic antioxidant defence mechanisms, since vitamin C is itself regenerated by glutathione (Meister 1992).

It has been reported that ascorbic acid is effective in preventing the oxidation of blood glutathione during exercise (Sastre et al 1992), thus illustrating a mutually dependent synergistic relationship between vitamin C and glutathione, allowing

vitamin C to continue to act as an effective free radical scavenger. Oxidation of blood glutathione however is frequently seen in exercise and as such is a commonly used index of exercise-induced oxidative stress (Gohil et al 1988, Ji and Fu 1992, Ji et al 1993).

A corollary to supplementing subjects with ascorbic acid is to examine the effects of an ascorbic acid depleting diet in relation to exercise performance. A reduced time to exhaustion during treadmill running in guinea pigs fed an ascorbic acid depleting diet has been reported (Packer et al 1986).

It has been has stated by Goldfarb (1993) that there is a need to investigate the role of vitamin C on exercise-induced lipid peroxidation and oxidative stress, and specifically, the effect of acute vitamin C supplementation on exercise-induced lipid peroxidation needs to be determined in humans. Therefore part of this thesis will address the lack of research in this area. Specifically the role of vitamin C, via an antioxidant intervention study, will be examined on exercise-induced free radical production from an ESR perspective. Additionally several indices will be used to assess the impact of acute ascorbic acid supplementation including: direct evidence using ESR spectroscopy; and supporting assay of free radical-mediated lipid peroxidation; as well as immunological assay.

To this authors knowledge, there are still no published studies specifically addressing these issues, particularly with regard to the use of ESR spectroscopy and exercising humans !

Chapter Three

ESR THEORY, METHODOLOGY AND PILOT STUDIES

3.0 Introduction to Electron Spin Resonance (ESR) Spectroscopy.

ESR is also known as Electron Paramagnetic Resonance (EPR) Spectroscopy. The term EPR was used when studies of transition metal ions were being undertaken while ESR was used to denote free radical studies. Nowadays both acronyms are used inter-changeably, although there is a trend to use the term Electron Magnetic Resonance (EMR) to bring it in line with nuclear magnetic resonance spectroscopy.

The seminal work of Stern and Gerlag in the 1920's demonstrated that a beam of silver atoms passing through a sufficiently strong inhomogenous magnetic field would split in two. This splitting is caused by the electrons in the outermost orbital possessing a property known as "spin". The spin of an electron described by the spin quantum number, S, yields an associated magnetic moment (dipole). These magnetic moments can interact with an applied magnetic field resulting in differing energy states.

Transition between energy states may be induced by absorption of a photon of appropriate frequency, which can be detected by ESR. Thus ESR detects the presence of the unpaired electron in the free radical by observing the resonance absorption of the microwave radiation required to reverse the direction of the magnetic dipole. The basic feature therefore of ESR is the ability to detect and characterise the presence of an unpaired electron in a sample.

ESR is widely used in chemical research where steady state concentrations of free radicals tend to be high. Its use however, is hampered in exercise physiology and clinical medicine by low steady state concentrations and the transient nature of the species concerned. The aqueous nature of these samples also cause difficulties due to the large decrease in instrument sensitivity observed when

samples are aqueous. This is caused by the ability of water to strongly absorb microwaves and "block" the ESR signal.

Two commonly used methods of overcoming these difficulties is the use of spin trapping agents and freezing of the samples usually in liquid nitrogen or helium. ESR is arguably the most direct, specific and sensitive technique to investigate paramagnetic materials. A modern spectrometer is theoretically capable of detecting about 10^{-9} M compared with NMR of about 10^{-1} M. However this is not routinely achieved and a more realistic detection limit of 10^{-7} M is possible practically for X-band spectrometers working at 9.5 GHz and 3 cm radiation.

3.1 Theory of Electron Spin Resonance (ESR) Spectroscopy.

In this thesis only a brief overview of ESR is given, the reader is directed to Atherton (1993) for a more detailed account. ESR spectra arise because an electron posesses spin, S (=1/2), and this spin has an associated magnetic moment (dipole) that is subject to quantum conditions. If an external magnetic field (H), is applied, the electron dipoles will orientate with (parallel) or against (anti-parallel), the magnetic field. Therefore the electron can exist in two energy levels which are known as the Zeeman energy levels.

3.1.1 Zeeman Energy Levels.

These are the two energy levels resulting from application of an external magnetic field, the difference between these energy levels is known as the Zeeman splitting. Since $\Delta E \propto H$, the difference between the two energy levels is directly proportional to the external magnetic field. Transitions between the two

Zeeman levels can be induced by the absorption of a photon of energy, hν, equal to the energy difference between the two levels.

Figure 3.1: Zeeman equation.

$$\Delta E = h\nu = g\beta H$$

where;

h = Planck's constant

ν = frequency of electromagnetic radiation

H = strength of external magnetic field in gauss

β = a constant known as the Bohr magneton (9.2733×10^{-28} J/gauss)

g = spectroscopic splitting factor or g factor (g=2.0023).

Figure 3.2: Zeeman energy levels of an electron in an applied magnetic field.

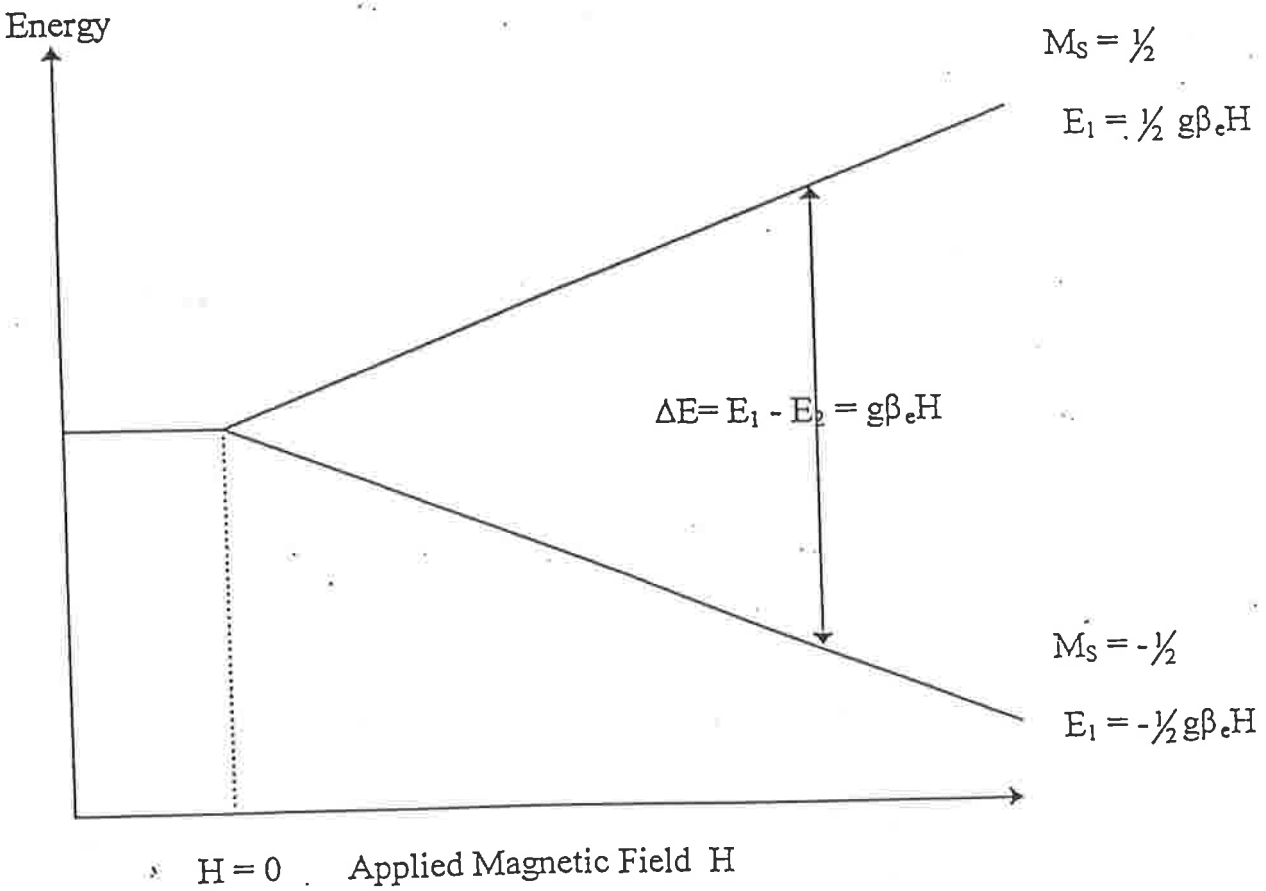


Figure 3.2 illustrates the transition that occurs when the degenerate energy levels for an electron are split by an external magnetic field into two levels.
It can be seen that the position of adsorption varies with magnetic field. Since ESR spectrometers may operate at different fields and frequencies eg. X-band is 9.5 GHz and Q-band 35 GHz, it is far more convenient to refer to the adsorption in terms of its g-value.

$$g = \frac{\Delta E}{\beta H} = \frac{h\nu}{\beta H}$$

The existence of two Zeeman levels, and the possibility of inducing transitions from the lower energy level to the higher energy level is the very basis of ESR spectroscopy.

The resonance experiment can be conducted in two ways; either the magnetic field is kept constant and the applied frequency varied, or, the applied frequency is held constant and the magnetic field varied. Generally in ESR spectroscopy the latter is usually the case since it is easier to vary the magnetic field over a wider range than to change frequency.

Practical advantages normally restrict magnetic fields to a maximum of 10,000 gauss and transitions occur at around 9.6 GHz. For organic radicals the magnetic field used is approximately 3,000 gauss (300 mT). This is a wavelength of approximately 3 cm which places the required frequency for energy transition in the microwave region of the electromagnetic spectrum ($\nu \cong 9$ GHz).

3.1.2 Relaxation.

If electrons were to be continually promoted from a low energy to a high energy level then the population of both energy levels would equalise and there would be no net absorption of radiation. In order to maintain a population excess in the lower level the electrons must be able to return to their low energy state. In order to do this the electrons must be able to transfer their excess spin energy either to other species or to the surrounding lattice as thermal energy.

The mechanism by which electrons transfer energy is known as the relaxation process, and the time taken for the spin system to lose 1/e of its excess energy is known as the relaxation time. Two relaxation processes are possible: spin-lattice relaxation and spin-spin relaxation.

(i) Spin-Lattice Relaxation.

The excess spin energy is transferred to the surrounding lattice by spin lattice relaxation. This relaxation is due to lattice motions such as molecular tumblings in liquids or gases.

(ii) Spin-Spin Relaxation.

The excess spin energy is transferred by interaction between paramagnetic centres from one molecule to another. This mode of relaxation is important when the concentration of the paramagnetic species is high, ie. the spins are close together.

3.1.3 Heisenberg's Uncertainty Principle.

If the relaxation time is too fast then the electrons will only remain in the upper state for a very short period of time. Heisenberg's Uncertainty Principle states that if a system exists in an energy state for a limited time Δt seconds, then the energy of that state will be uncertain. As Δt decreases then the uncertainty of the resonance magnetic field increases, this gives rise to broadening of the ESR signal as shown below:

$$\Delta E \times \Delta t = h = \frac{h}{2\pi}$$

3.1.4 Broadening of ESR Lines.

(i) Lattice Relaxation.

The line broadening effect of lattice relaxation can be reduced by working at lower temperatures since this reduces thermal fluctuations, thus increasing the lifetime of the excited state ΔT, and hence reducing ΔH.

(ii) Spin-Spin Relaxation.

If the concentration of paramagnetic species in the sample is high then they can interact more effectively with each other causing spin-spin relaxation. Biomolecular oxygen is a common paramagnetic species which causes spin-spin relaxation leading to broadening of the spectral line. Line broadening can be reduced by removing oxygen from the system and using lower concentrations of radicals. In biological systems the concentration of radicals is generally very low

therefore the only recourse necessary to prevent line broadening is to remove oxygen.

3.1.5 Integrated Intensity.

Due to the rapid relaxation processes the ESR signal intensity is directly proportional to the number of spins (radicals) in the sample. It is conventional that ESR spectra be shown as a first derivative of the absorption signal. This is achieved by the application of a secondary alternating sinusoidal magnetic field. This is known as a phase locked detection system and greatly enhances the signal-to-noise ratio.

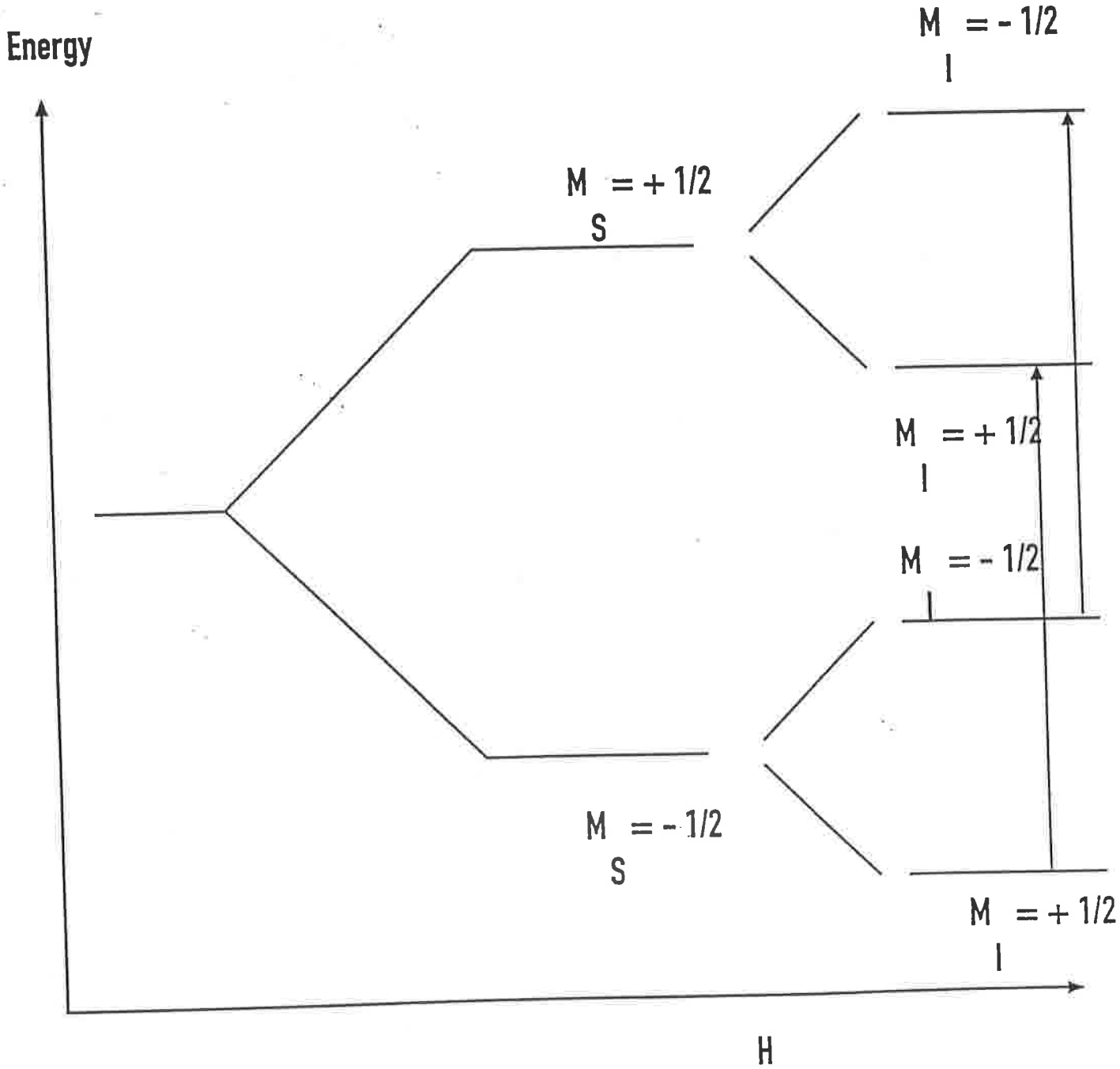
3.2 Hyperfine Structure.

If the interaction of the electron with an applied external magnetic field were the only effects detectable by ESR then all spectra would consist of a single line and would be of little interest to researchers. However, the most useful information that can be derived from an ESR spectrum usually results from nuclear hyperfine structure. The source of this hyperfine structure is the interaction of magnetic nuclei within the radical with the magnetic moment of the unpaired electron.

Many molecules contain nuclei which have a magnetic moment and thus spin angular momentum, and these can interact with the electron and split its energy levels still further to give hyperfine structure. The spin of a nucleus is described by the spin quantum number I. Nuclei with magnetic moments include $H^{\cdot}$, and C^{13}. In the case where an odd electron interacts with one proton, the magnetic field of the proton produces a small additional field which adds to or subtracts from the external magnetic field. Each level in figure 3.3 is further split into two by interaction with a proton. Nuclear spins do not change when the electron

changes levels and therefore, transitions occur between half the levels, ie. those having the same nuclear spin. Thus two transitions can occur and the electron that produced the single line in the ESR spectrum now produces a pair of lines.

Figure 3.3: Interaction between unpaired electron and nucleus.



Examples of radicals that would yield a two line spectrum include the hydrogen atom ($H^{\cdot}$) and dichloromethyl radical ($^{\cdot}CHCl_2$). The energy difference between the two nuclear Zeeman levels is lower than that of electron Zeeman levels. The magnetic moment of an unpaired electron can now not only interact with the applied external magnetic field but also with local nuclear magnetic moments. It is this interaction between electron and nuclear magnetic moments which which give rise to hyperfine structure.

Generally, a single nucleus splits an ESR line into 2nI+1 lines, where I is the nuclear spin. For a hydrogen atom where I is 1/2, a single proton produces two lines. When n equivalent protons interact with the electron the spectrum consists of n + 1 lines, the intensity of which varies in a binomial fashion which can be predicted by the application of Pascal's triangle (see figure 3.4 below).

Figure 3.4: Pascal's triangle.

			1					No equivalent hydrogens
		1		1				1 equivalent hydrogens
	1		2		1			2 equivalent hydrogens
1		3			3		1	3 equivalent hydrogens
1	4		6		4		1	4 equivalent hydrogens.

For example, the methyl radical with three protons should give a spectrum of four lines in the ratio of 1:3:3:1. Two types of electron spin - nuclear spin interactions must be considered, those of an isotropic and anisotropic nature.

3.2.1 TYPES OF ELECTRON SPIN-NUCLEAR SPIN INTERACTION.

(i) ISOTROPIC INTERACTIONS.

Isotropic interaction is a quantum interaction related to the finite probability of the unpaired electron to be at the nucleus, and is termed the Fermi contact interaction. The isotropic interaction concerns exclusively s-type orbitals or orbitals with partial s character since these orbitals only have finite probability density at the nucleus. The spherical symmetry of s-orbitals accounts for the isotropic nature of the contact interaction.

An example of isotropic hyperfine interaction is the hydrogen atom. The electron spin is interacting with the proton (I = 1/2) spin. This later may assume two possible orientations with $M_I = \pm 1/2$. Thus the nuclear magnetic moment further splits each Zeeman level into two sub-levels. The ESR selection rule ($\Delta Ms = \pm 1/2$) allows only two transitions and therefore, the ESR spectrum of the hydrogen atom is composed of two resonance lines separated by approximately 508 gauss (50.8 mT).

NB:
The spacing between the lines is known as the hyperfine coupling (splitting) constant and changes as a function of the trapped radical, this aids identification and characterisation of the radical.

(ii) ANISOTROPIC INTERACTIONS.

Anisotropic electron-nuclear (hyperfine) couplings are due to the di-polar interaction between the nuclear and electron magnetic moments when the unpaired electron is in non-spherically symmetrical orbitals (p, d and f orbitals).

Anisotropic interaction is heavily dependent upon the relative orientation of the magnetic nucleus and the electron.

Anisotropic interactions are particularly important in solid state ESR. However in solution, the paramagnetic species rotate and tumble exploring all possible orientations relative to the applied field, thus averaging out all anisotropic dipole-dipole interactions to zero.

3.3 ESR SPIN TRAPPING THEORY.

It is not usually possible to observe free radicals directly in biological systems. There are several reasons for this:

a. The rapidity with which highly reactive free radicals react with cellular constituents.
b. The low steady state concentration of the radicals.
c. The high water content of most biological samples including blood, and tissues such as muscle and liver absorb the microwaves, preventing detection by the spectrometer.

In order to overcome these drawbacks a technique known as spin trapping is frequently used in biological investigations. Spin trapping was developed by Janzen and Blackburn (1968), the basic concept involves the introduction of a diamagnetic compound, the spin trap, to harvest the paramagnetic radical to a stable spin adduct (see figure 3.5 overleaf):

Figure: 3.5: Interaction between a diamagnetic spin trap and a paramagnetic species.

ST	+	$R^{\cdot}$	=	SA
diamagnetic		paramagnetic		paramagnetic
(spin trap)		(transient free radical)		(long lived spin adduct)

The stable paramagnetic spin adduct then accumulates until it is observable by ESR. One of the main advantages of the spin trapping technique is that by determination of the parameters of the spin adduct spectrum it is often possible to identify the nature of the primary trapped radical, or at least determine the type of radical trapped.

In addition to this, the stability of the trapped adduct permits detection by ESR, thus extending the capabilities of ESR to detect radicals that would otherwise be unobservable.

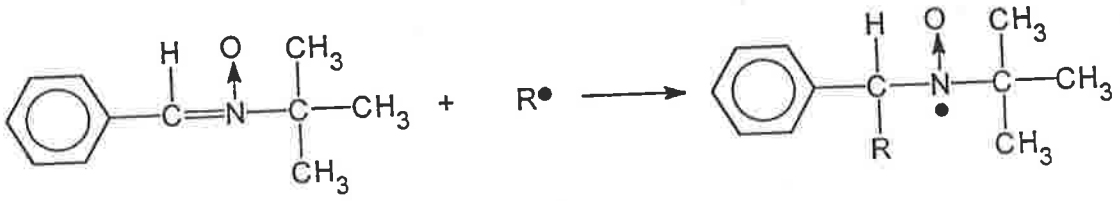
Two main categories of spin traps are currently used;

1. Nitrones.

Probably the most commonly used nitrones are α-phenyl-tertbutyl-nitrone ($C_{11}H_{15}NO$) (PBN) and 1,1, 5-dimethylpyrroline-N-oxide (DMPO) ($C_6H_{11}NO$). Of these, PBN appears to be the most widely used, reasons for its popularity include its comparatively long shelf stability, it has been commercially available for many years and was the first nitrone to be used in this manner. It is also both thermally and photochemically stable (Dr Gary Buettner - personal communication). A disadvantage of PBN is that it does not readily distinguish

between radicals since its ESR spectra generally consists of a triplet of doublets with a relatively small variation in the doublet splitting as a function of the trapped radical.

Figure 3.6: The interaction between PBN and a transient radical.



The radical bonds onto the carbon atom adjacent to the nitrogen and so gives comparatively little information about the nature of the trapped radical. However there are extensive collections of hyperfine coupling constants (hfc) for many PBN adducts which aid identification and characterisation (see table 3.0 below).

TABLE 3.0: EXAMPLES OF COUPLING CONSTANTS FOR PBN SPIN ADDUCTS.

Radical trapped	Nitrogen hfc/gauss	Hydrogen hfc/gauss
CCl_3·	14.0	1.8
Phenyl·	16.2	4.3
Superoxide	14.3	2.3
Ethyl·	16.2	3.4
Hydroxyl	15.3	2.8

Perhaps the primary advantage that another frequently used nitrone ie. DMPO, has over PBN is its ability to yield comparatively more information about the trapped radical. The variation in coupling constants achieved with DMPO as a function of the trapped radical may permit easier identification of the radical.

However, a major criticism of DMPO is its renowned instability and ease with which artifactual signals are produced. In addition to this DMPO must be purified prior to use whereas PBN can be used without further purification (Dr. Gary Buettner - personal communication).

2. Nitroso compounds.

The compound 2-methyl-2-nitrosopropane (MNP) is probably the most frequently used nitroso spin trap. Nitroso spin traps have an inherent advantage over nitrones for radical identification in that the added group lies immediately adjacent to the nitroxide centre and therefore can easily yield additional hyperfine splitting. A disadvantage is that they are far less stable both thermally and photochemically than their nitrone counterparts. This instability therefore causes frequent artifactual signals, they are also unreliable in trapping oxygen-centred radicals.

The experimental procedure used in spin trapping experiments is dependent upon a number of factors including the manner of radical production, inertness of any solvents used, and the type of deoxygenation required. In most cases bubbling of nitrogen gas is sufficient to deoxygenate the sample prior to analysis, however in certain cases where for example concentrations of radicals are very low, degassing using a freeze-thaw vacuum pump procedure may be required. In addition to this, removal of water is often a pre-requisite for detection by ESR in biological systems and therefore organic extraction using a suitably inert non-polar solvent is also required.

3.3.1 SPIN ADDUCT DECAY.

A number of decay routes exist for spin adducts the most common being reduction of the spin adduct.

ie. $-N-O^{\cdot} + H^{\cdot} \rightarrow -N-^{\cdot}OH$

The above equation illustrates reaction of the nitroxide with a hydrogen atom. It is worth noting that the observation of this reaction is increasingly common due to the increased use of spin traps in biological systems where a multitude of potential reducing agents exist. A common reductant in biological systems is ascorbate and thus the rapid decay of the signal may well be a function of the ascorbic acid concentration.

A positive aspect of this is that it is unlikely that any signals detected will be artifactually high since the presence of ascorbate in the system would prevent an increase. It is possible to oxidize nitroxides, however this appears to be less common than reduction in biological systems.

In summary, the main priorities following successful detection by ESR of a spin trapped radical is identification and determination of the origin of the species. In complex biological systems where there may exist several possible mechanisms of free radical production and several sites of origin, this may not always be possible. In order to overcome this, supporting assays should be used so that conclusions can be made regarding the origin and indentity of the signal. Spin trapping nevertheless, provides a powerful technique for the study of free radical production and observation by ESR in biological systems (Anderson-Evans 1979).

3.3.2 Spin Trapping Sample Extraction Procedure.

Listed below is the procedure developed and used in this research:

1. A 140 mmol.L^{-1} solution of the spin trap α-phenyl-tert butyl nitrone (PBN) (Sigma Ltd. Dorset, UK) in de-ionised water of the highest purity available, was freshly prepared on the morning of the experiments. The PBN was used without further purification.
2. 0.5ml of normal saline was added to 1ml of the spin trap solution and inserted into glass 6ml serum separation tubes (Becton-Dickinson Ltd. Oxford UK). The vacutainers were then kept on ice in the dark until use (approximately 20 minutes).
3. The vacutainers containing the spin trap solution were then used to take blood samples from an ante-cubital vein immediately before and after the exercise tests.
4. Immediately blood was taken, the serum separation tubes containing the blood and spin trap solution were inverted gently to mix the contents.
5. Tubes were then immediately placed on ice or in the refrigerator in the dark to allow to clot. Clotting normally occurred at approximately 10 minutes.
6. On clotting, the tubes were immediately centrifuged for 10 minutes at 3500 rpm. The tubes were then placed in an ice filled dewar, in the dark, and transported to the ESR laboratory for immediate analysis.
7. Organic extraction was performed using HPLC grade toluene (Sigma-Aldrich) without further purification, having been previously scanned by ESR for the presence of paramagnetic species.
9. An equal volume of toluene was added to the serum/adduct and vortex mixed for 30 s. The volumes were 1.0 ml of toluene to 1.0 ml of plasma.

10. The sample was then centrifuged for 3 minutes to separate the organic layer.
11. The organic layer containing the PBN adduct was pipeted into a 22 cm precision bore quartz ESR sample tube.
12. The sample was then vacuum de-gassed in a freeze-thaw procedure, using either a mercury diffusion pump or a turbo pump to 10^{-3} Torr for 4 repeated cycles. A Pirani 14 gauge was used to monitor pressure change.
13. The samples were immediately analysed at room temperature using a JEOL RE2X series X-band spectrometer.
14. Blank experiments were also carried out which included: the ESR analysis of the empty cavity on the JEOL spectrometer; an empty quartz ESR tube; and the chemicals used in the work.
15. Duplicate samples were taken at the same time in order to carry out validatory assays eg. malondialdehyde, lipid hydroperoxides (except in the pilot studies).

3.4 HAEMATOLOGICAL MEASUREMENTS.

3.4.1 BLOOD SAMPLING.

All blood samples were taken on the same day of the week at the same time of day by the same investigator in an attempt to control for biological variation (Reilly 1994), and also to minimise inter-subject analytical variation. Furthermore diet has been shown to exert effects on several blood borne metabolites in particular plasma lipids and lipoproteins (Pronk 1993), consequently all blood samples were taken after an overnight fast.

3.4.2 Collection of venous blood.

Each subject assumed a seated position and was allowed to rest for 5 minutes before blood letting. A tourniquet was secured to the right arm with the minimum constriction required to obtain a blood sample (Bachorik 1982). Blood samples were obtained from an ante-cubital forearm vein and analysed for a variety of biochemical parameters as described below.

3.5 Lipid Peroxidation Assays.

MDA and LH were assayed using the following techniques:

A. Malondialdehyde (MDA).

The method of Young and Trimble (1991) was employed to measure MDA. This HPLC method with fluorometric detection overcomes the lack of specificity generally associated with the measurement of MDA.

Blood was collected from an ante-cubital vein in di-potassium ethylene diamine tetra-acetic acid (EDTA) vacutainers. The thiobarbituric acid (TBA) reaction was carried out by mixing 250µL of 1.22 M phosphoric acid, 450µL of HPLC grade water, 50µL EDTA plasma, and 250µL of 0.44 M TBA. MDA standards are prepared using 1,1,3,3,-tetramethoxypropane which yields equimolar amounts of MDA under the reaction conditions. The reaction mixture is then heated in a boiling water bath for 1 hour in sealed glass tubes and cooled to 4°C in ice. The mobile phase consists of 50% methanol and 50% 25mM phosphate buffer at pH 6.5. The flow rate is 0.8mL/min. Samples are neutralized and de-proteinized before injection onto the column by mixing 200µL of plasma (or standard) with

360μL HPLC grade methanol and 40μL 1M NaOH solution. The excitation was at 532 nm and the emission was at 553 nm.

B. Lipid Hydroperoxides (LH).

The ferrous-oxidation xylenol orange (FOX) assay was used to determine blood LH concentration (Nourooz-Zadeh 1994, Wolff 1994). Blood was collected from an ante-cubital vein into 6 ml serum separation tubes (Becton-Dickinson Ltd. UK).

The selective oxidation of ferrous to ferric ion by hydroperoxides in dilute acid yields ferric ions that may be detected with ferric sensitive dyes. A blue-purple coloured complex is produced with the selective binding of xylenol orange (XO, o-cresosulfonephtalein 3'3-bismethylimino di-acetic acid, sodium salt) to the ferric ions produced. The absorption can then be measured at 560nm.

$$Fe^{3+} + XO \rightarrow \text{blue-purple complex (560nm).}$$

Two "recipes" for the FOX assay exist, and the second recipe, listed below, is suitable for the determination of LH in blood. This recipe is more appropriate due to the small concentrations of LH found in blood and the relatively high concentrations of non-peroxidized background lipids. The reason for this is that alkoxyl radicals generated during the ferrous oxidation step react with native lipid generating further hydroperoxides in a chain reaction. Inclusion of a suitable chain breaking antioxidant such as butylated hydroxy toluene (BHT) overcomes this difficulty by repairing alkyl radicals produced by the reaction of alkoxyl radicals with unsaturated lipids.

The protocol for determination of LH in human blood using the FOX assay is as follows:

1. Chemicals used in FOX assay;
   100 μM xylenol orange
   250 μM ammonium ferrous sulphate
   90% HPLC grade methanol (used to solubilize blood lipids)
   4mM BHT
   25mM H_2SO_4.
2. 50 μl of serum is added to 950 μl of the above mixture.
3. The sample is vortex mixed and allowed to incubate at room temperature for 30 min.
4. Centrifugation is performed to remove protein.
5. Absorbance is read at 560nm.

The possibility that loosely available iron yielded artifactual results is overcome by the addition of triphenylphosphine (TPP) to serum samples and incubating at room temperature for 30 min. prior to the addition of the FOX2 reagent (Nourozz-Zadeh 1994, Wolff 1994).

Serum hydroperoxides can thus be determined directly by monitoring absorbance changes at 560nm before and after the addition of TPP. This approach obviates the difficulty caused by the reaction of serum ferric ion with xylenol-orange.

3.6 Total Antioxidant Capacity.

The method used is that based on enhanced chemiluminescence (Whitehead et al 1992). Light emission occurs when the chemiluminescent substrate luminol is

oxidized in a reaction catalysed by horseradish peroxidase in the presence of the enhancer para-iodophenol. The addition of para-iodophenol causes a prolonged and more stable light emission. The continuous output of light is dependent on the presence of free radical intermediates derived from oxygen. The significance of this is that light emission is therefore sensitive to interference by free radical scavenging antioxidants, particularly chain breaking antioxidants. Light emission is restored when the added antioxidant has been consumed in the course of the reaction.

The assay procedure for this technique is as follows:

1. Chemicals used were from an antioxidant detection pack (Amersham Ltd.) Signal reagent consists of assay buffer, tablets A and B containing luminol, para-iodophenol, and horseradish peroxidase (HRP).
2. 20µl of HRP is added to 5ml de-ionised water. Dilution to optimum luminescent output is then performed.
3. A calibration solution of the water soluble tocopherol analogue Trolox is prepared at 80µmol.l^{-1} .
4. Test solutions are prepared by diluting the blood plasma sample by 1:10 with de-ionised water.
5. 800µl of de-ionised water is added to the luminometer cuvette, followed by 100µl of signal reagent and 100µl of HRP conjugate solution and thoroughly mixed.
6. The cuvette is placed in the luminometer and the light emission allowed to stabilise.
7. 20µl of the sample is added to the cuvette and a timer started simultaneously.
8. Light output will be quenched immediately due to the presence of antioxidants such as tocopherol and ascorbic acid.

9. The assay is allowed to continue until suppression of the reaction.
10. The time in seconds is recorded until light output recovers to 10% of the initial light output.
11. This time is compared to the time value for the Trolox calibrant and the total antioxidant capacity expressed as $\mu mol.l^{-1}$ Trolox equivalents.

Note: Solutions of the water-soluble tocopherol analogue Trolox are stable at room temperature for several hours making it suitable as a standard. The Trolox calibrant ($80\mu mol.l^{-1}$) is added to the reaction undiluted. Almost complete suppression of light emission is obtained for a given period of time (t), after which the output is rapidly restored giving a square-wave appearance. Chemiluminescent reactions containing 20µl of standard and of typical intensity result in t values of approximately 250s. The t value is related linearly to the amount of standard antioxidant present which enables a standard plot to be constructed. This then enables the antioxidant capacity of solutions to be determined, and expressed in terms of $\mu mol.l^{-1}$ Trolox equivalents. The normal concentration for plasma TAC in young, healthy male students is 447.1 ± 60.2 $\mu mol.L^{-1}$ Trolox equivalents (mean ± SD) (Whitehead et al 1992).

3.7 MEASUREMENT OF PLASMA ASCORBIC ACID.

The method used to measure plasma ascorbic acid concentration is that based on the kinetics of fluorescence development by condensation of dehydroascorbic acid with 1,2-phenylenediamine (Vuilleumier and Keck 1989). Blood samples were collected in 3.5ml glass vacutainers containing ethylene-diamine-tetra-acetate (EDTA) as an anti-coagulant, immediately pre and post-exercise. The samples were centrifuged as previously mentioned and 100µL of EDTA plasma was added to 900µL of 5% metaphosphoric acid immediately post-centrifugation.

The stabilised samples were then placed on ice and deposited in a -80°C freezer within 1 hour until analysis. In this assay a 5% solution of metaphosphoric acid is used to de-proteinate the sample. This was freshly prepared on the morning of the experiment, and kept on ice until use. A 2M buffer of pH 6.2, consisting of sodium acetate trihydrate and acetic acid is also prepared. The stock solution consists of 2mg ascorbate oxidase in 1ml acetate buffer. The oxidizing reagent consisted of 0.1ml stock solution diluted with 10ml buffer. A coupling reagent consisting of 20mg of 1,2-phenylenediamine in water, and a standard solution consisting of 0.5mg pure ascorbic acid (Hoffman-La Roche) per ml of 5% metaphosphoric acid are used in the assay. This assay is sensitive and reproducible for the measurement of total plasma ascorbic acid and is suitable for the analysis of ascorbic acid in biological fluids (Vuilleumier and Keck 1989).

3.8 Measurement of plasma retinol, α-tocopherol, β-carotene, α-carotene, lycopene, and β-cryptoxanthin.

The method used for the simultaneous determination of the above mentioned antioxidants is based on HPLC (Thurnham et al 1988). EDTA plasma (0.25mL) mixed with 0.25mL sodium dodecyl sulphate is deproteinized with ethanol containing tocopherol acetate, and extracted with heptane containing 0.5g BHT per litre. The sample is vortex mixed and then centrifuged to separate the phases.

The heptane supernatant (0.7mL) is removed and evaporated under nitrogen at 40°C and the residue re-constituted in 0.25mL of mobile phase (acetonitrile/methanol/chloroform). The sample is then analysed using HPLC at 320nm and 292nm using tocopherol acetate as an internal standard.

3.9 PROCEDURE FOR MEASUREMENT OF PLASMA ENDOTOXIN (LIPOPOLYSACCHARIDE) CONCENTRATION.

Plasma endotoxin was measured using the Limulus amebocyte lysate (LAL) assay (Novitsky 1994). The method uses a kinetic LAL assay (KQCL, Bio-Whittaker, UK). Standard endotoxin was dissolved in pyrogen-free water (PFW) yielding a concentration of 50 Eu.ml $^{-1}$, which was then vortex mixed for 5 min. Dilutions for the standard curve were performed in pyrogen-free glass tubes, 1:10 serial dilutions of the endotoxin standard in PFW were done to give 5.0, 0.5, 0.05, and 0.005 Eu.ml $^{-1}$. 50 µl of each standard and sample were placed in duplicate wells of a multititre plate and then placed in the holder of the plate reader which had been previously warmed to 37° C. Spiked recovery was then performed.

3.10 ANTHROPOMETRIC MEASUREMENTS.

Each subject was weighed prior to the exercise test. The subjects were instructed to wear shorts and t-shirt and remove footwear. The subjects were weighed using a balanced weighing scales (Seca, Cardiokinetics, Salford UK) which was calibrated with a 5.0 kg free weight prior to each "weigh in". The subjects height was measured using a stadiometer (Seca, Cardiokinetics, Salford UK), the accuracy of which was checked with a tape measure.

3.11 CARDIOVASCULAR AND RESPIRATORY MEASUREMENTS.

3.11.1 LABORATORY HEART RATE.

An electrocardiography calibrated short-range telemetry system (Polar Ltd. Kenilworth, UK) was used to monitor heart rate during the exercise tests. Heart

rates were sampled and averaged every 5 s, and transmitted via electromagnetic waveforms to a watch receiver. These data were downloaded using a Polar Advantage Interface™ connected to a computer and used in the overall analysis.

3.11.2 Measurement of oxygen uptake (VO_2).

The oxygen uptake/consumption of the subjects was measured using a MedGraphics CPX/D on-line gas analysis system (Cardiokinetics, Salford UK) which was previously calibrated against a Douglas bag system. Subjects breathed through a broad flanged rubber mouthpiece that was connected to a low resistance (<5cm H_2O at 300 $L.min^{-1}$), low dead space (<50ml) Salford two-way non-rebreathing respiratory valve. Expired airflow was directed to a pneumotachograph via 1.5 m of tubing. The MedGraphics CPX/D on-line gas analysis utilises a breath-by-breath measurement technique. This analyser incorporates a pneumotach system consisting of a "pre-Vent" pneumotach (valve dead space = 20 ml) and transducer. This was calibrated at 5 different flow rates using a 3 L syringe to verify a linear response prior to experimentation. Signals were directed to a waveform analyser which converts the analog signals to correlate with flow. A computer integrated flow relative to time to obtain a volume measurement. Dried expired gas was presented to fast-responding infra red CO_2 and zirconium O_2 analysers which were calibrated using a high quality reference gas (21% O_2, balanced N_2) and a calibration gas (5% CO_2, 12% O_2, balanced N_2, BOC Special Gases). Respiratory parameters expressed at BTPS were sampled every 30 s and printed on-line (Citized Swift 200 UK).

3.12 Protocol for maximum oxygen uptake test.

This is described in detail in study 1. Briefly however, all subjects commenced cycling at 120 W (2.0 kg) workload and 60 rpm. The test is incremental and

progressive and workload was increased by 30 W every 3 minutes until exhaustion. The test is designed to elicit peak oxygen uptake.

3.13 Protocol for Wingate anaerobic exercise test.

A standard anaerobic exercise test (Wingate test) was also performed at the Human Performance Laboratory using the same cycle ergometer, in order to test the effect of anaerobic metabolism of free radical production. It is described in detail in study 2. Briefly however, the test involves a 30 s supra-maximal sprint against resistance calculated relative to bodyweight. The resistance is calculated using the formula of 0.75g/kg bodyweight and is applied after initial inertia and unloaded frictional resistance is overcome (Bar-Or 1987).

Subjects were required to cycle to 60 rpm while the weight basket was held, thus overcoming unloaded frictional resistance, following a count of three the basket was released and the subject cycled as fast as possible for 30 s while being verbally encouraged.

3.14 Measurement of whole blood lactate concentration.

Approximately 50 µl of venous blood was collected into a capillary tube (Analox). The tubes contain heparin, fluoride and nitrite to stabilise blood lactate concentrations. The tube was immediately placed on an electric mixer for 4 minutes. Each sample rotates approximately 180° every 5 seconds ensuring adequate mixing and preventing coagulation. The concentration of blood lactate was measured using an automated electrochemical analyser (Analox PGM7, London, UK). Fresh buffer was added to L-lactate:oxidoreductase at an ambient temperature of 21° C and entered into the analyser. Injection of a sample into the

cuvette activates an oxidation-reduction reaction catalysed by L-lactate oxidoreductase at a pH of 6.5. The maximum rate of oxygen consumption during the reaction is directly related to the concentration of lactate in the sample.The analyser was calibrated prior to blood sampling and a quality control material (Analox lactate/pyruvate quality control serum) used to ensure reagent reactivity. Samples were analysed in duplicate and a mean of the two results used.

3.15 Measurement of haematocrit.

A venous blood sample was obtained and collected into a heparinised capillary tube (Hawksley and Sons Ltd. Sussex, UK). The distal end of the tube was sealed with cristaseal (Hawksley and Sons Ltd. Sussex, UK) and inserted into a Micro Haematocrit Centrifuge (Hawksley and Sons Ltd. Sussex, UK) with the sealed end facing outwards. The blood sample was immediately centrifuged at 11, 800 revolutions per minute (RPM) for 5 minutes and the length of the column of packed erythrocytes measured using a Hawksley Micro Haematocrit Reader. The value obtained was then corrected for haemoconcentration induced by exercise using the Van Beaumont equation (Van Beaumont 1972).

3.16 Dietary analysis.

Subjects were required to complete a 3-day dietary diary. Information was analysed using a commercially available software package (Comp-Eat, Nutrition Systems, London, UK).

3.17 Statistical analysis.

Descriptive as well as inferential statistical tests were employed in the analysis of these data. Both parametric and non-parametric tests were used to analyse the differences between **pre-exercise** and **post-exercise**. Non-parametric statistical tests are used when there is noticeable heterogeneity of the sample (Altman 1991), as appeared to be the case with these data. The paired samples t-test and Wilcoxon signed rank test for paired data were the parametric and non-parametric equivalent tests respectively (Kinnear and Gray 1994). The relationship between two dependent variables was ascertained by a correlation test. Analysis was performed using the SPSS software package (Surrey, UK) and Minitab. Statistical difference was defined as $p < 0.05$ for all tests.

3.18 Pilot studies.

The literature has shown that in general measurable increases in free radical concentrations or products of oxidant damage such as malondialdehyde, occur only during strenuous exhaustive exercise. Therefore in order to maximize the possibility of producing sufficient quantities of free radicals to allow detection by ESR the exercise test must be sufficiently strenuous. In addition to the centrality of the exercise test, the optimal spin trapping protocol should be determined so that any free radicals produced by exercise will be detectable by ESR.

3.18.1 Pilot study 1: Physiological assessment of exercise stress.

Three (n=3) subjects performed an aerobic exercise test to volitional exhaustion. The tests were carried out on the same calibrated cycle ergometer that the main studies used (Model 824ε, Monark Ltd. Stockholm, Sweden), and were repeated three times at fortnightly intervals.

The objective of this experiment was to identify physiological parameters associated with exhaustive exercise. During the test the subjects heart rate (HR) was monitored continually using a portable heart rate telemetry device (Polar Sport Tester, Polar Ltd. Kenilworth, UK). In addition to this, subjective assessment of exercise stress was made via continual reference to the ratings of perceived exertion (RPE) scale which has been previously validated (Borg 1982) (Appendix 1). Subjects were required to maintain a constant 60 rpm and resistance was increased by 0.5kg (30 watts), every three minutes until the subject could no longer continue despite verbal encouragement. At this stage maximum heart rate and rating of perceived exertion was determined, the subject was then deemed to be exhausted. The combination of heart rate and ratings of perceived exertion allow quantification of the level of physiological stress an exercise test exerts on a subject.

Table 3.0 and 3.1 below show the results of the physiological assessment of exercise stress. It can be seen that maximum age predicted heart rate was achieved and furthermore that the exercise was perceived as being very strenuous by reference to the Borg scale.

TABLE 3.0: RESULTS OF PILOT STUDY 1.

	Subject 1 Test:			Subject 2 Test:			Subject 3 Test:		
	1	2	3	1	2	3	1	2	3
Max. resistance (kg)	4.0	4.5	4.5	5.0	5.0	5.0	5.0	5.0	5.0
Time (min)	13.39	17.20	16.48	19.51	19.48	19.00	20.10	20.49	19.01
Max. heart rate (bpm)	175	196	192	186	188	190	189	195	192
Max. RPE	19	17	19	18	19	19	19	19	19
Age	29			28			28		
Height (cm)	174.3	174.4	174.3	178.5	178.4	178.6	173.0	173.1	173.1
Weight (kg)	72.3	72.5	72.6	83.0	83.1	84.0	73.3	73.0	73.4

TABLE 3.1: SUMMARY OF RESULTS OF PHYSIOLOGICAL ASSESSMENT OF EXERCISE STRESS.

	Time (min)	Max HR (bpm)	RPE (arbitrary units)	Age (years)	Height (cm)	Weight (kg)	Resistance (kg)
Subject 1	15.69± 1.17	187±12	18.33± 0.66	29	174.3± 0.03	72.5±0.14	4.33± 0.16
Subject 2	19.30± 0.16	188±2	19.00± 0.00	28	178.5± 0.06	83.4±0.31	5.0±0.00
Subject 3	19.86± 0.44	192±3	19.00± 0.00	28	173.1± 0.03	73.2±0.12	5.0±0.00

NB: Results are expressed as mean ± SEM; Max HR, maximum heart rate; RPE, rating of perceived exertion.

DISCUSSION OF PILOT STUDY 1: PHYSIOLOGICAL ASSESSMENT OF EXERCISE STRESS.

Maximum heart rate as a function of age can be predicted according to the work of Robinson (1938). This prediction of maximum age predicted heart rate (220 bpm - age) is presented with the standard deviation of ± 10 bpm. Thus age predicted maximum heart rate was achieved in all subjects. Achievement of maximum heart rate is suggestive of maximal oxygen convection to the tissues. However the amount of oxygen flux through the tissues is also influenced by diffusion of gases.

It was decided in view of these results that a minimum heart rate of 180 bpm, irrespective of age, must be achieved in order to ensure sufficient exercise stress on the subject and thus maximal oxygen flux through the tissues. Use of the ratings of perceived exertion scale (Borg 1982) indicate that the exercise test was sufficiently taxing to induce the perception of very hard, strenuous exercise. The fact that the subjects could not continue despite vigorous verbal encouragement is suggestive of exhaustion.

CONCLUSION.

Results of this study indicate that this aerobic exercise protocol can be used in the main studies and is sufficient to induce exhaustion and maximum heart rate, by definition therefore, this will also elicit a peak or maximum whole body oxygen uptake. The potential thus exists for a large and measurable increase in the concentration of free radicals in the venous circulation.

3.18.2 PILOT STUDY 2: ESR SPIN TRAPPING PILOT STUDIES.

Prior to attempting any spin trapping studies the ESR spectra of untreated whole blood and serum was determined using a quartz aqueous flat cell. Additionally the ESR spectra of a blank tube and empty JEOL cavity was examined (Appendices 2 and 3 respectively). Samples were analysed at room temperature on a JEOL RE2X series X-band spectrometer fitted with a TE 110 cavity, under the following operating conditions: Temperature 300 K, frequency 9.4250 GHz, power 10 mW, field centre 334.7, sweep width ± 5.000 mT, scan rate 4.0 min, modulation width 0.1000 mT, time constant 0.03 sec. See figure 3.8.

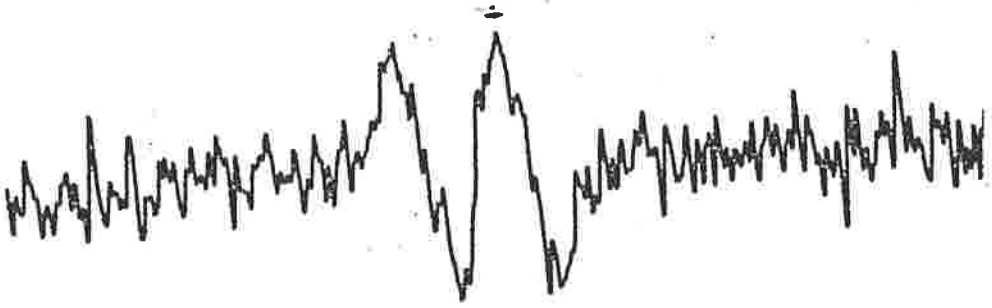
NB: Ethical approval and written informed consent was obtained prior to participation (Appendices 4 and 5 respectively).

FIGURE 3.8: TYPICAL ESR SPECTRA OF WHOLE BLOOD.

-----> H

0.129 MT

FIGURE 3.9: TYPICAL ESR SPECTRA OF UNTREATED HUMAN SERUM.



——> H

0.129 mT

Figure 3.9 ESR operating conditions: Temperature 300 K, frequency 9.3890 GHz, power 10 mW, field centre 333.6 mT, sweep width 1.500 mT, scan rate 8.0 min, modulation width 0.1000 mT, gain 4000, time constant 0.30 sec.

Figure 3.8 shows the resting ESR spectra of untreated whole blood with no evidence of any radical species. Figure 3.9 shows the resting ESR spectra of untreated human serum/plasma and clearly illustrate the presence of the ascorbate free (ascorbyl) radical (a_H = 0.183 mT). This species is unequivocally assigned as the ascorbate free radical following the work of Sharma and Buettner (1993) who reported an identical plasma signal with a_H = 0.18 mT and also Sommani and Arroyo (1995) who reported a signal with a_H.= 0.189 mT. This is to be expected since ascorbic acid is present in human blood in concentrations ranging from 10-90 $\mu mol.L^{-1}$ (Levine et al 1996). Ascorbate is reversibly oxidized forming the ascorbate free radical which in turn is oxidized to dehydroascorbic

acid, dehydroascorbic acid may be reduced back to ascorbic acid with the formation of the intermediate ascorbate free radical (Levine et al 1996). No other paramagnetic species were evident.

ESR SPIN TRAPPING USING DIMETHYLPYRROLINE-N-OXIDE (DMPO).

The spin trap DMPO (Sigma Ltd. Dorset, UK) was dissolved in de-ionised water at a concentration of 10 $mg.ml^{-1}$ whole blood. 1.5 ml of the DMPO solution was added to a 3 ml glass vacutainer containing the anticoagulant heparin (Becton-Dickinson Ltd. Oxford, UK). Three (n=3) subjects performed an aerobic exercise test to volitional exhaustion as described previously. The blood samples were analysed in a JEOL RE2X series X-band spectrometer in an aqueous quartz flat cell and precision bore quartz ESR tube, using the following operating conditions: Temperature 300 K, frequency 9.4200 Ghz, Power 10 mW, 335.2 mT, sweep (scan width) 7.500 mT, scan time 5.0 min, modulation width 0.1000, gain 4000, time constant 0.10 sec.

RESULTS OF PILOT STUDY 2.

No radical species were detected in these samples.

TABLE 3.2: PHYSIOLOGICAL RESULTS OF EXERCISE TEST.

	Time (min)	Max HR ($beats.min^{-1}$)	RPE	Resistance (kg)
Subject 1	17.50	189	19	4.5
Subject 2	19.20	185	19	5.0
Subject 3	20.10	197	19	5.0

NB: Max HR, maximum heart rate; RPE, rating of perceived exertion.

DISCUSSION OF PILOT STUDY 2: DMPO AND EXERCISE.

No radicals were present pre or post-exercise. The subjects exercised to exhaustion and were unable to continue despite verbal encouragement. Slight haemolysis was observed in one of the samples. The possibility exists that the concentration of DMPO was not high enough to trap any radicals produced since in biological systems of this nature the concentration of radicals will usually be very low.

CONCLUSION.

It was determined therefore that in order to maximize the possibility of trapping any radicals produced, the concentration of the spin trap be increased to 20 mg.ml. The experiment was thus repeated using an increased concentration of DMPO.

3.18.3 PILOT STUDY 3: THE EFFECT OF INCREASED SPIN TRAP CONCENTRATION ON EXERCISE-INDUCED FREE RADICAL PRODUCTION.

The concentration of DMPO was increased from 10mg.ml^{-1} to 20mg.ml^{-1} of whole blood and the subjects repeated an identical exercise test. ESR conditions were as stated previously.

RESULTS OF PILOT STUDY 3:

Please see table 3.3 overleaf.

TABLE 3.3: PHYSIOLOGICAL RESULTS OF EXERCISE TEST USING INCREASED SPIN TRAP.

	Time (min)	Max HR (beats.min^{-1})	RPE	Resistance (kg)
Subject 1	17.07	181	19	4.5
Subject 2	19.41	182	19	5.0
Subject 3	20.00	193	19	5.0

NB: Max HR, maximum heart rate; RPE, rating of perceived exertion.

ESR RESULTS.

No paramagnetic species were detected in these samples.

DISCUSSION OF PILOT STUDY 3: THE EFFECT OF INCREASED SPIN TRAP CONCENTRATION.

No radicals were evident using this concentration of spin trap. Subjects exercised to exhaustion and heart rates approximated age predicted maximum heart rate.

CONCLUSION.

It was again determined that an increase in the concentration of spin trap from 20mg.ml^{-1} to 30mg.ml^{-1} whole blood would be justified in spite of slight haemolysis being observed in two of the six samples. In addition to this the possibility exists that any radicals initially trapped were decaying before analysis. In an attempt to overcome this the blood will be immersed in liquid nitrogen immediately on sampling.

3.18.4 Pilot study 4: The effect of increased spin trap concentration and sample freezing on exercise-induced free radical production.

The experiment was repeated using an increased concentration of DMPO from 20mg.ml^{-1} to 30mg.ml^{-1}. The same three (n=3) subjects repeated an identical aerobic exercise test to volitional exhaustion. ESR analysis was performed under the same operating conditions. In addition to this duplicate samples were taken and immediately frozen in liquid nitrogen to attempt to preserve any radicals trapped.

Results of pilot study 4.

Table 3.4: Physiological results of exercise test using increased spin trap concentration and freezing of samples.

	Time (min)	HR (beats.min^{-1})	RPE	Resistance (kg)
Subject 1	16.39	185	19	4.5
Subject 2	18.57	181	19	4.5
Subject 3	19.30	188	19	5.0

NB: Max HR, maximum heart rate; RPE, rating of perceived exertion.

ESR results.

No paramagnetic species were detected in these samples.

DISCUSSION OF PILOT STUDY 4: THE EFFECT OF INCREASED SPIN TRAP CONCENTRATION AND SAMPLE FREEZING IN LIQUID NITROGEN.

Following the several unsuccessful attempts at detecting free radicals in human serum using ESR and the spin trap DMPO a different technique was employed, ie. immersion of the vacutainer in liquid nitrogen immediately on sampling. The objective of this was to attempt a further stabilisation of any trapped radicals by slowing the reaction rate kinetics.

Immersion in liquid nitrogen resulted in several of the vacutainers shattering presumably as a result of thermal shock which necessitated repeating the experiment. The experiment was repeated using plastic instead of glass vacutainers, a major drawback of this is that the plastic commonly used in clinical settings such as syringes, has been shown to produce artifactual ESR signals (Buettner et al 1991). However while the vacutainers did not shatter, all the samples were haemolysed.

CONCLUSION.

Despite haemolysis no free radical signal was detected using ESR. It was also noted that on exposure to ambient light the DMPO solution would take on the appearance of a pale green colour which was assumed to be indicative of photolytic degradation. DMPO has been reported to react with oxygen-centred radicals notably, $O_2^{\cdot -}$ (Finkelstein et al 1980), which was the basis for its original use. However, a relatively low rate constant (10 $M^{-1}.s^{-1}$.) (Finkelstein et al 1980), combined with the susceptibility to undergo degradation reactions and air oxidation (Sanders et al 1994), coupled with the short lifetime of DMPO spin adducts (Buettner 1993), led to a reassessment of the research.

RATIONALE BEHIND THE CHANGING OF SPIN TRAPS.

The lack of success in the experiments thus far led to a new approach being pursued. The spin trap DMPO was replaced by α-phenyl-tert-butylnitrone (PBN).

The use of PBN confers several advantages over DMPO including:

1. Stability, PBN is known to be a relatively stable compound;
2. As a solid it is not overly sensitive to light, oxygen or water;
3. In inert solvents, such as toluene or benzene, any photolytic degradation that occurs does not produce significant concentrations of nitroxide radicals;
4. It is relatively water-soluble and soluble to fairly high concentrations (~ 0.1M) in many solvents;
5. Purity, commercially available PBN is normally of sufficient purity to exclude the need for further purification;
6. Perhaps most importantly of all, the spin adducts of PBN are comparatively stable (Janzen 1971, Finkelstein et al 1980).

A disadvantage with the use of PBN however is the fact that the ESR spectrum almost always consists of a triplet of doublets with little variation in the hyperfine (splitting) coupling constants, as such only a small amount of information regarding the nature and structure of the trapped radical can be obtained.

3.18.5 Pilot study 5: Exercise-induced free radical production: The ability of PBN to trap blood-borne radicals.

Given the lack of success in previous studies and the low concentrations of free radicals found in biological systems, it was decided to maximize the possibility of trapping free radicals by having a saturated solution of PBN in the glass vacutainers. Due to previous observations of haemolysis in the samples preliminary studies were carried out in order to determine the effect of PBN on blood.

Results showed that PBN dissolved in de-ionised water led to haemolysis being observed in six out of eight (75%) samples examined. The addition of physiological saline in a 2:1 volume abolished any observable evidence of haemolysis. Two (n=2) subjects performed an identical aerobic exercise test to volitional exhaustion. The ESR conditions were as previously stated.

Results of pilot study 5:

Table 3.5: Physiological results of exercise test using PBN.

	Time (min)	Max. HR (beats.min^{-1})	RPE	Resistance (kg)
Subject 1	17.51	185	19	4.5
Subject 2	18.09	181	19	4.5

NB: Max HR, maximum heart rate; RPE, rating of perceived exertion.

ESR RESULTS OF PILOT STUDY 5.

No paramagnetic species were detected in these samples.

DISCUSSION OF PILOT STUDY 5: THE ABILITY OF PBN TO TRAP BLOOD-BORNE RADICALS DURING EXERCISE-INDUCED OXIDATIVE STRESS.

Given that the PBN was at a saturated concentration and previous evidence of the strenuous nature of the exercise test, it was determined that since blood is an aqueous solution the presence of water molecules (and possibly oxygen), known to absorb microwaves, may be "blocking" the ESR signal.

CONCLUSION.

An organic extraction and de-gassing technique will now be employed using the inert, non-polar solvent toluene (HPLC grade), to remove water and oxygen from the samples before ESR analysis.

3.18.6 PILOT STUDY 6: THE EFFECT OF ORGANIC EXTRACTION ON THE ELECTRON SPIN RESONANCE SIGNAL OF PBN ADDUCT PRE AND POST-EXERCISE.

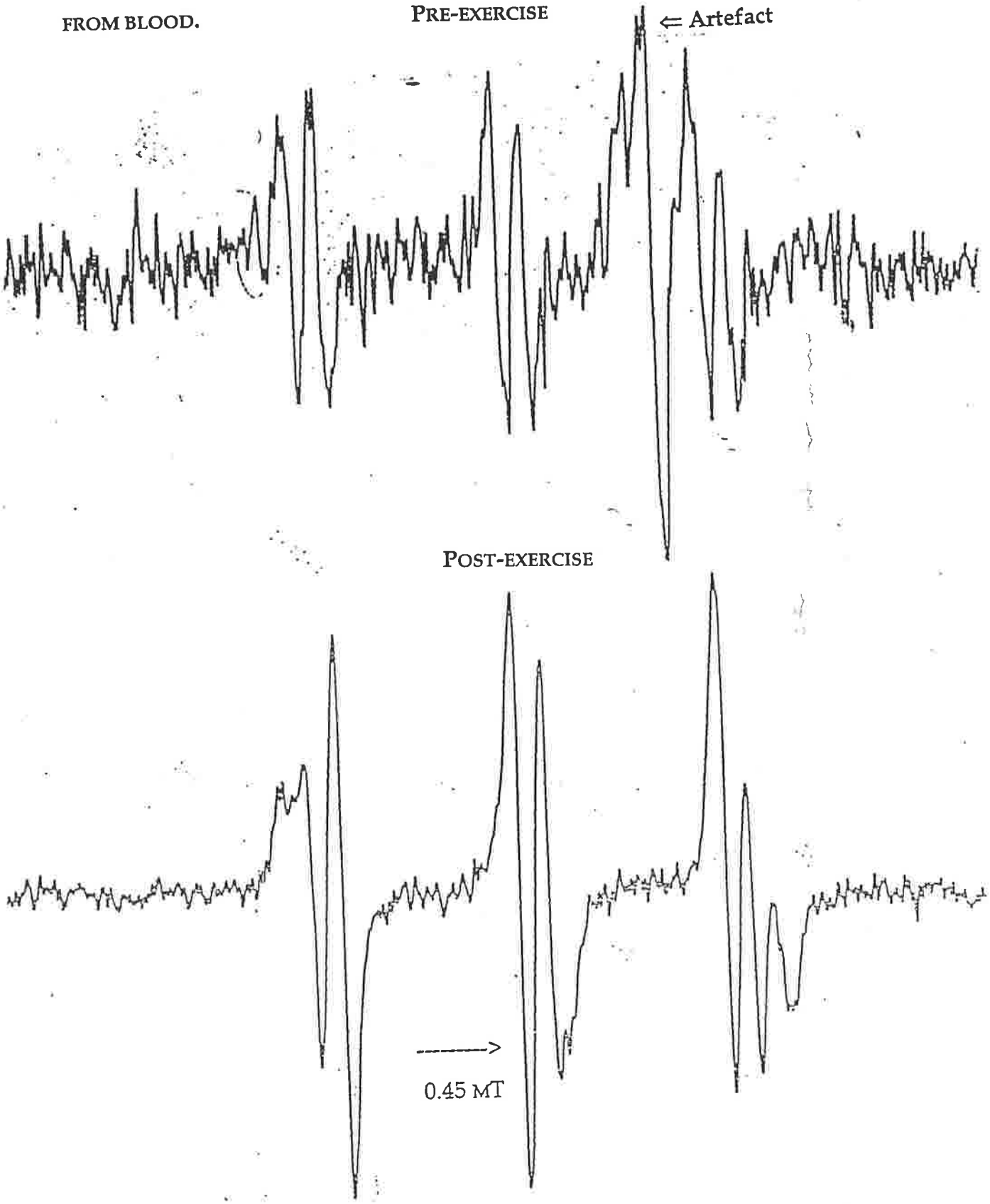
One subject (n=1) performed an identical, progressive aerobic exercise test to volitional exhaustion on the same calibrated cycle ergometer.

RESULTS OF PILOT STUDY 6:

TABLE 3.6: PHYSIOLOGICAL RESULTS OF EXERCISE TEST USING PBN AND AN ORGANIC EXTRACTION TECHNIQUE.

	Time (min)	Max. HR (beats.min^{-1})	RPE	Resistance (kg)
Subject 1	16.51	193	19	4.5

FIGURE 3.10: ESR SPECTRA OF PBN ADDUCT FOLLOWING ORGANIC EXTRACTION FROM BLOOD.



DISCUSSION OF PILOT STUDY 6: THE EFFECT OF ORGANIC EXTRACTION ON THE ESR SIGNAL PRE AND POST-EXERCISE.

The above spectra clearly show evidence of a triplet of doublets characteristic of a nitroxide adduct of PBN. There also appears to be an increase in the signal intensity pre vs. post-exercise. However analysis of the spectra suggests that there are two species present and yields the following tentative hyperfine coupling constants: species 1, a_N = 1.6 mT, a_H = 0.223 mT approximately 75% of the signal and species 2, a_N = 1.6 mT, a_H = 0.425 mT approximately 25% of the signal. There is some degree of uncertainty regarding the identity of the radicals due to overlap of the spectra, species 1 may however be tentatively ascribed to an oxygen-centred radical eg. superoxide (a_N = 1.43, a_H = 0.23 mT) or hydroxyl (a_N = 1.53, a_H = 0.28 mT). Species 2 may be identified as a PBN/$CO_2^{\cdot-}$ adduct (a_N = 1.60 mT, a_H = 0.457 mT) which has been previously reported in biological fluids (Hughes et al 1991).

Additionally there appears to be an 'extra' line in the ESR spectrum of the pre-exercise sample. This was investigated and found to be an artefactual line caused by a scratch on the ESR tube (please see appendix 6). It is now essential to repeat the study with a larger number of subjects since this result may be due to chance.

3.18.7 PILOT STUDY 7: THE EFFECT OF EXHAUSTIVE AEROBIC EXERCISE ON FREE RADICAL PRODUCTION: A PILOT STUDY.

Five (n=5) healthy male subjects aged 18-30 years volunteered for the study. They were required to perform the same exhaustive aerobic exercise test which would elicit maximal oxygen uptake.

RESULTS OF PILOT STUDY 7:

TABLE 3.7: PHYSIOLOGICAL RESULTS OF PILOT STUDY 7.

	Time (min)	Max. HR (beats. min^{-1})	RPE (arbitrary units)	Resista-nce (kg)	Height (cm)	Weight (kg)
Subject 1	16.52	188	19	4.5	174.4	72.5
Subject 2	16.22	179	19	4.5	167.2	55.8
Subject 3	17.55	187	19	4.5	172.7	71.2
Subject 4	17.19	192	19	4.5	184.6	84.3
Subject 5	19.23	191	19	5.0	178.5	88.0
Mean ± SEM	17.34 ± 1.9	187 ± 8	19.00 ± 0.00	4.6 ± 0.10	175.48 ± 9.91	74.36 ± 14.67

NB: Max HR, maximum heart rate; RPE, rating of perceived exertion.

The above table 3.7 show the mean and group results for this study. It can be seen that the subjects exercised for an average of approximately 17.5 minutes. This resulted in increased heart rate approximating to 220-age, which is indicative of maximal cardiac output and hence maximal whole body oxygen uptake. It is important to note that this aerobic exercise test is to exhaustion. The strenuous nature of the test can be further indicated by high scores on the Borg ratings of perceived exertion scale, signifying very hard exercise.

FIGURE 3.11: TYPICAL ESR SPECTRA OF PBN ADDUCT IN HUMAN SERUM.

PRE-EXERCISE

POST-EXERCISE

--------> H

0.45 MT

Hyperfine coupling constants: a_N 1.370 mT; a_H 0.192 mT.

DISCUSSION OF PILOT STUDY 7.

The above ESR spectra (figure 3.11) illustrate a triplet of doublets characteristic of a PBN nitroxide adduct and show a clear increase post-exercise. The hyperfine coupling (splitting) constants are a_N 1.370 mT and a_H 0.192 mT. This would identify the radicals as being oxygen- or carbon-centred (Buettner 1987).

Given the overlap in coupling constants it is difficult to categorically identify the radical especially when there exists *in vivo* numerous possible sources of radicals (see chapter 2). It is however suggested that the species are carbon-centred or alkoxyl radicals both of which may be formed by secondary reactions of initial or primary oxygen radicals with membrane lipids and yield very similar coupling constants (a_N = 1.36 mT, a_H = 0.156 mT), to those reported by Garlick et al (1987) in reperfused rat hearts. This suggestion is further supported by almost identical coupling constants (a_N = 1.36 mT and a_H = 0.19 mT) found in the blood of patients undergoing elective cardioplegia reported by Tortolani et al (1993). Furthermore formation of these radicals may also result in increased products of lipid peroxidation such as malondialdehyde and lipid hydroperoxides due to propagation. This hypothesis is further tested in study 1 (chapter 4). The species would thus be secondary radicals formed as a consequence of primary radical attack such as hydroxyl radical ($^{\cdot}OH$), or superoxide anion ($O_2^{\cdot-}$) on the cell membrane leading to further propagation of radical species and consequent lipid peroxidation. It is however difficult to categorically state the identity of the trapped species and therefore caution is advised when attempting to attribute an observation to a specific pathway of production.

The spin trap PBN was used in this study since its relative lipophilicity will allow penetration into cells; it has the ability to trap oxygen-centred radicals in biological systems; commercially available PBN is of high purity; and the PBN

adducts are comparatively stable (Charlon and Leiris 1988). In addition to this the stability and lack of toxicity of PBN in oxygenated perfusate has been established (Charlon and Leiris 1988), this is particularly important since the post-exercise blood samples will have been exposed to large amounts of oxygen. Furthermore 5,5 dimethyl-1-pyroline-N-oxide (DMPO) is unsuitable for many biological uses due to the instability of its adducts (Samuni et al 1989).

A key issue however that must be born in mind despite published work, is that free radicals are by definition transient species and thus although PBN adducts are stable when compared to the adducts of DMPO, the short lifetime of adducts precludes extensive use of signal averaging (Buettner and Kiminyo 1992), in view of this ESR spectra were scanned for 20 minutes. Despite these drawbacks ESR spin trapping is currently the best available technique for the study of radicals in biological systems (Pou et al 1989).

The ESR spectra show a triplet of doublets pre-exercise which reflect resting levels of free radical production. At rest, aerobic respiration and mitochondrial electron transport continually occurs, thus providing a ready source of free electrons to participate in radical generating reactions as suggested in chapters one and two. A noticeable increase post vs. pre-exercise is suggestive of a causative role of exhaustive exercise in free radical production. Many workers have reported increases in the products of lipid peroxidation as well as changes in antioxidant status such as glutathione post-exercise, thus reflecting the oxidative stress induced by exercise.

These novel pilot ESR data corroborate previously reported animal studies in the literature. Further investigations are needed regarding the importance of the type of exercise required to generate increased free radical production for example, aerobic vs. anaerobic exercise. The significance of this is that during

strongly anaerobic exercise whole body oxygen uptake is not raised significantly when compared to a maximal oxygen uptake test. This would allow conclusions to be drawn regarding the role of whole body oxygen flux in the genesis of exercise-induced free radical production.

Furthermore the effect of antioxidant supplementation in relation to exercise-induced oxidative stress needs to be elucidated. Much work has been performed examining the role of vitamin E in exercise-induced oxidative stress since it is a an important chain breaking antioxidant. Few studies however have been performed regarding the role of ascorbic acid in this context, and there appear to be no studies regarding vitamin C and ESR in relation to exercise-induced oxidative stress. An advantage of using ascorbic acid as an intervention is that it is readily measurable by ESR in human blood. During the pilot studies a variety of exercise tests were performed and blood sampling was carried out during as well as pre and post-exercise, all of these failed until the introduction of the organic extraction technique.

CONCLUSION.

This work will now be further investigated and repeated with a larger sample number. Additionally several different validatory or supporting assays will be incorporated in order to gain insight into the possible mechanism of production and the role of whole body oxygen uptake in relation to exercise-induced free radical production.

3.19 QUALITY CONTROL STUDIES.

In addition to the pilot studies reported, a number of quality control experiments were also performed. Including allowing the samples to 'sit on the bench' for up to 60 minutes before analysis in order to determine the stability of the sample. It

was noted that the sample remained stable for approximately 40 minutes and then start to decay. Immersing the samples in an ice-filled dewar preserved the ESR signal intensity for up to 2 hours (data not presented). Additionally the reliability of the spectrometer was assessed using known concentrations of the synthetic radical TEMPO to identify any discrepancy in the signal intensity.

Chapter Four

Study 1:

AEROBIC EXERCISE AND FREE RADICAL PRODUCTION

4.0 INTRODUCTION.

It has been reported that maximal aerobic exercise leads to increases in the post-exercise ESR signal intensity possibly as a consequence of increased oxygen flux (Ashton et al 1996). However, a criticism of that research was the lack of supporting or validatory assays. Therefore, the present study was designed to examine the effects of aerobic exercise eliciting maximal oxygen uptake on *in vivo* free radical production. Human volunteers performed a progressive incremental aerobic exercise test to exhaustion on a calibrated cycle ergometer while undergoing breath-by-breath oxygen uptake measurement. The test is designed to elicit maximum whole body oxygen uptake. The biological significance of this is that aerobic exercise produces a three-fold increase in the ESR signal intensity in the muscle of rats exercised to exhaustion (Davies et al 1982). To this author's knowledge there is no comparable data on the effects of exhaustive aerobic exercise on the ESR signal in human sera.

It follows that as oxygen consumption and hence oxidative stress is increased, free radical production also increases (Clarkson 1995). This is important since, if an exercise test involves conraction of large muscle groups and elicits a large increase in heart rate and oxygen uptake, it can be assumed that oxygen flux through the tissues also increases. Furthermore in muscular activity lasting more than ten minutes over 90% of the required ATP is provided by the aerobic system, there also exists a linear relationship between heart rate and oxygen uptake (Astrand and Rodahl 1986, Tortora and Grabowski 1996). This is demonstrated by increased cardiac output during exercise which may rise to 40 $L.min^{-1}$ (Newsholme et al 1994). The increase in oxygen uptake during exercise can be greater than 50 fold in active skeletal muscle (Newsholme et al 1994). Consequently the flux of electrons down the mitochondrial electron transport chain also increases. The resulting leakage of electrons possibly forming reactive

oxygen species (ROS) particularly the hydroxyl radical ($^{\cdot}OH$), superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (Halliwell and Gutteridge 1989), is also magnified. The 2-5% of electrons that leak during resting metabolism is then also raised several fold (see section 2.1.1). Recently however it has been suggested that the percentage of electrons leaking out of the electron transport chain may be as high as 15% (Sawyer 1988). However, providing more oxygen for mitochondria will not necessarily speed up electron transport, due to the very high affinity of cytochrome oxidase for oxygen, but it will cause greater electron leakage and superoxide formation (Gutteridge and Halliwell 1994). The potential for a large and measurable increase in exercise-induced free radical concentration thus exists.

Damage to skeletal muscle and peripheral tissues can accompany exercise and this may be free radical-mediated (Davies et al 1982, Jackson et al 1985). The major membranes of skeletal muscle appear to incur damage by acute strenuous exercise and damage to the sarcolemma is inferred by the appearance in the blood of enzymes normally found in muscle such as: lactate dehydrogenase; pyruvate kinase; and myoglobin (Schwane et al 1983). Delayed onset muscle soreness, which normally peaks approximately 2-3 days post-exercise is indicative of damage to skeletal muscle following severe exhaustive exercise. This is however more commonly associated with eccentric muscle contraction (Armstrong 1990), although it is worth stating that most dynamic exercises such as running - particularly downhill, cycling and box stepping have eccentric components.

Mitochondrial damage is indicated by loss of respiratory control, decreases in coupling of energy synthesis to oxygen consumption, and perhaps most importantly increases in lipid peroxidation (Davies et al 1982). Such changes are observed in isolated mitochondria from exhaustive exercised rats compared to

rested rats. In peripheral tissues and erythrocytes, changes in osmotic fragility have been reported, as have changes in the ratio of reduced to oxidized glutathione (Sen et al 1994).

Severe exhaustive exercise, like stress, causes immunosuppression which may increase susceptibility to infection (Weiss 1989). There is as yet little knowledge of the cumulative effects of long term exercise, the effects on critical cellular systems are also not well known although it is suspected that repeated damage may influence longevity and health.

This study seeks to demonstrate an increase in the ESR signal intensity (amplitude) in human serum following maximal aerobic exercise, allowing inferences to be made regarding the importance of whole body maximal oxygen uptake on exercise-induced free radical production. In addition to this a range of indirect supporting biochemical parameters will be measured providing further insight into the possible mechanism of increase in the ESR signal and these are summarised in the methods section of this chapter.

4.1 Methodology.

Study design.

Twelve (n = 12) healthy male subjects volunteered for the study. They consisted primarily of a cross-section of the student population of the University of Wales Institute, Cardiff (UWIC). Suitable members of staff also participated where necessary. They were aged between 18 and 30 years and were free of any physician-diagnosed disease established by means of a questionnaire (Appendix 7). All except one subject were non-smokers. Subjects who took vitamin

supplements were excluded. The study was cross-sectional in design in an attempt to glean as much information as possible about exercise-induced free radical production in the general population. However sample size would limit inferences made. One consequence of this is that there was variation in volume of oxygen used by the subjects during the tests and in the other parameters examined demonstrating the heterogeneity of the sample.

Written informed consent was obtained prior to participation and ethical approval was granted by the South Glamorgan Local Research Ethics Committee as in the pilot studies. The tests were carried out in the morning after an overnight fast, subjects were asked to avoid breakfast on the morning of the test. In the days prior to the test the subjects were instructed to maintain their usual dietary and lifestyle pattern but were asked to refrain from exercise for 24 h before the test.

EXERCISE PROTOCOL AND BLOOD SAMPLING.

The exercise tests were carried out at the Human Performance Laboratory at UWIC. All exercise tests were performed on the same calibrated Monark cycle ergometer model 824ε (Monark Ltd. Stockholm, Sweden). All blood samples were drawn from an ante-cubital vein using a vacutainer system (Becton-Dickinson Ltd. Oxford, UK). The laboratory was temperature and humidity controlled (21° C, 45 %), respectively. The author attended a phlebotomy course prior to commencement of the research.

EXERCISE PROTOCOL FOR ACHIEVEMENT OF MAXIMAL OXYGEN UPTAKE (VO_{2MAX}).

Subjects were required to cycle at 60 ± 2 rpm until exhaustion on a mechanically braked cycle ergometer. Subjects were familiarised with the test in the week prior

to taking the test and the seat and handlebar settings noted. All subjects commenced cycling at 120 W resistance, equivalent to 2 kg mass on the flywheel, and the resistance was increased by 30 W (0.5kg) every three minutes until the subject could no longer continue despite verbal encouragement. An electric cooling fan was placed immediately in front of the ergometer and was turned on after 5 minutes of exercise.

MEASUREMENT OF OXYGEN UPTAKE (VO_2).

Measurement of ventilatory and pulmonary gas exchange was performed at the Human Performance Laboratory (UWIC), using the Medgraphics Cardiopulmonary CPX/D system, (Cardiokinetics, Salford, UK) as described in detail in section 3.11.2. The system was calibrated at 5 different flow rates using a 3 L syringe to verify a linear response prior to experimentation (Please see appendix 8, for example of calibration curve).

CRITERIA FOR ACHIEVEMENT OF $VO_{2MAX.}$

The subject was deemed to be exhausted when:

1. Respiratory exchange ratio (RER) ≥ 1.15 at termination of test.
2. He could no longer continue to maintain 60 RPM despite verbal encouragement (56 rpm was the cut-off point).
3. His heart rate equalled or exceeded the age predicted maximum heart rate, 220 bpm - age in years at termination of test.
4. Plateauing of the oxygen uptake curve where observed.

NB. Heart rate was continually monitored during the test via a portable heart rate telemetry system (Polar Ltd. Kenilworth, UK).

BLOOD SAMPLING.

Blood was collected from an ante-cubital vein immediately before and immediately after the exercise test via two venepunctures using a vacutainer system. For the resting pre-exercise sample, subjects were instructed to sit quietly in a chair for 5 minutes before the blood sample was taken. Immediately following the exercise test subjects were removed from the ergometer and the second (post-exercise) blood sample was taken. The time delay was no greater than 5-10 s post-exercise, as measured by stopwatch, although some delay was inevitable experimentally.

Blood was collected using the following vacutainers and analysed for free radical presence and evidence of lipid peroxidation using methods described in chapter three. In addition to this a range of individual blood antioxidant parameters were measured as detailed below. Exercise-induced haemoconcentration was measured by changes in haemotacrit level. All blood samples are pre and post exercise and include: malondialdehyde (MDA); ascorbic acid; α-tocopherol; β-carotene; α-carotene; lycopene; retinol; and total antioxidant capacity (TAC) from EDTA plasma. While lipid hydroperoxides (LH) and ESR were measured from serum samples, and endotoxin from plasma in specific pyrogen-free vacutainers. All blood samples were centrifuged at 3500 rpm for 10 minutes and immediately decanted into small Eppendorf tubes and stored at -80°C for a maximum of 6 weeks prior to analysis.

Since this work is principally a novel ESR investigation and since free radicals are by definition transient species, the ESR samples were always, without exception, analysed the same day with minimum time delay between collection and analysis. Upon centrifugation the ESR samples were kept in the vacutainers and placed in ice in the dark prior to analysis. Where appropriate samples were

allowed to clot prior to centrifugation. Therefore in the interests of standardisation all samples were immediately placed in the refrigerator for 10 minutes prior to centrifugation.

STATISTICAL METHODS.

Parametric and non-parametric statistical tests were used to analyse the data as described in section 3.17. There was agreement between the parametric and non-parametric tests. The reported p-values are for the non-parametric Wilcoxon signed ranks test tests since in study 2 there were n=7 subjects. It is most unlikely that these data would be normally distributed with this number of subjects thus in the interests of consistency non-parametric p-values are reported throughout the work.

4.2 RESULTS OF STUDY 1: AEROBIC EXERCISE AND FREE RADICAL PRODUCTION.

TABLE 4.0: ANTHROPOMETRIC AND NUTRITIONAL DATA.

Height (cm)	Body mass (kg)	BMI	Age (years)	Energy (kcals)	Fat (grams)	Protein (grams)	CHO (grams)
178.3 ± 10.0	77.4 ± 23.7	26.3 ± 1.3	24.6 ± 4.0	2105 ± 106	71.0 ± 2.39	88.0 ± 21.5	297.0 ± 47.1

NB: Results are expressed as mean ± SEM. BMI, body mass index (kg/m^2); CHO, carbohydrate. (Please see appendix 9 for example of dietary analysis).

Table 4.0 demonstrates the anthropometric and nutritional data from the cohort. It can be seen that there is a wide variation in the results which reflects the heterogeneity of the sample group. Nutritional data appears to suggest that

energy intake is slightly low compared to recommendations (DoH 1991). This may reflect student lifestyle.

TABLE 4.1: CARDIOVASCULAR AND HAEMATOLOGICAL DATA.

	VO_2 uptake (ml.kg. min^{-1})	Max heart rate (beats .min^{-1})	Time to exhaustion (min^{-1})	RER	Haem-atocrit % PCV
Pre-Exercise	6.73±1.08	67 ± 2	N/A	0.80± 0.04	44.4 ± 1.2
Post-Exercise	49.40 ± 2.73 **p=0.002**	191 ± 2 **p=0.002**	16.17± 1.01	1.23± 0.03 **p=0.01**	45.8 ± 2.3 **ns**

NB: Results are expressed as mean ± SEM, p-value for Wilcoxon signed rank matched pairs test; RER, respiratory exchange ratio; ns, not significant.

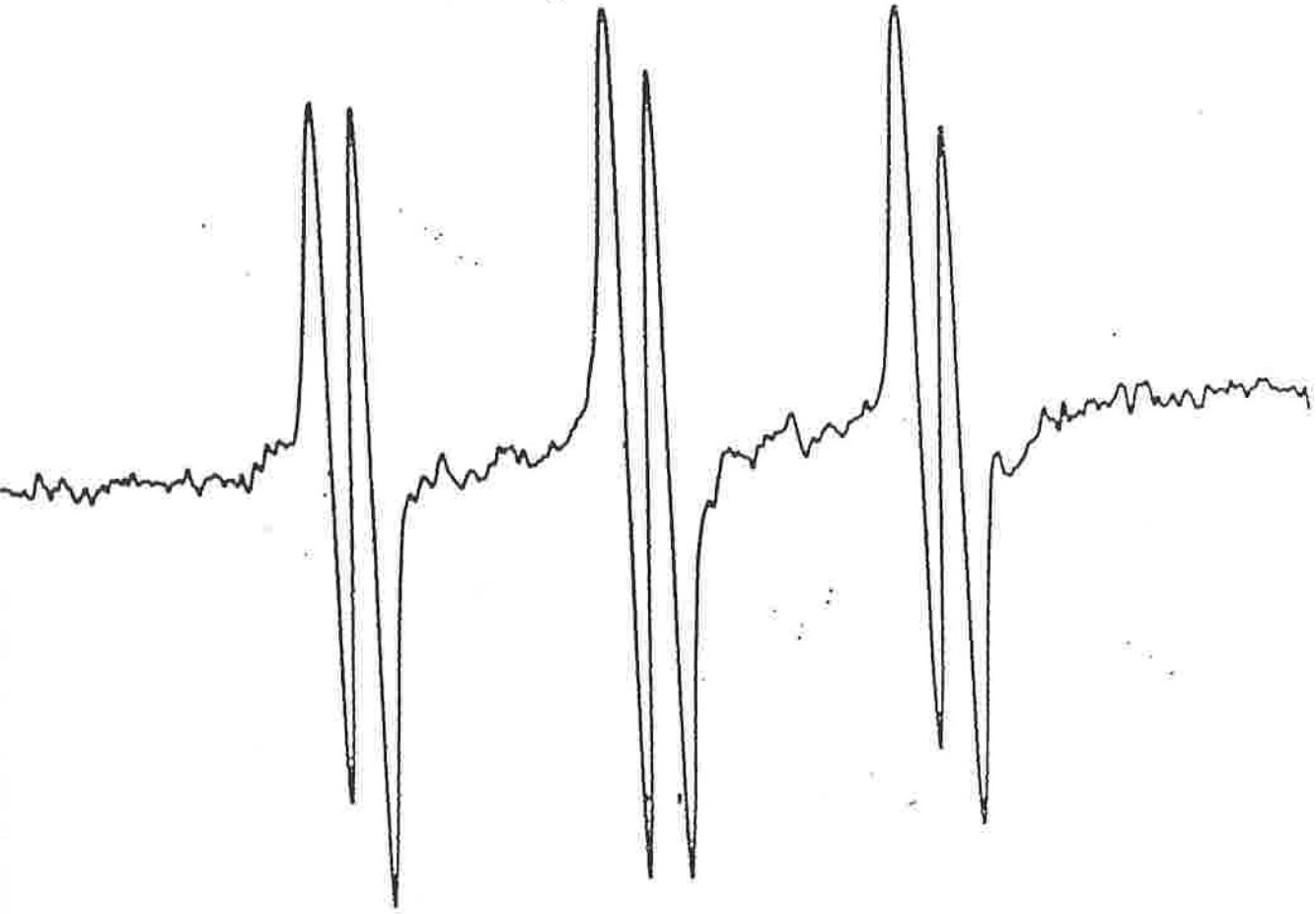
Table 4.1 presents the cardiovascular and haematological data from the study. There is a statistically significant increase in VO_2 following exhaustive aerobic exhaustive. This represents increased whole body oxygen uptake and concomitant tissue oxygen flux. These data are corroborated by significantly increased heart rate post-exercise, which also indicates increased tissue oxygen flux via increased cardiac output. Respiratory exchange ratio also increases post-exercise indicating metabolic changes. Exercise-induced haemoconcentration was measured by haematocrit changes which also increased non-significantly post-exercise. This may suggest changes in plasma volume (Van Beaumont 1972).

Figures 4.0 and 4.1 show typical ESR spectra of the PBN adduct in human serum and the ascorbate free radical respectively. It can be seen that exercise induces increase in the intensity of the ESR signal. This is interpreted as being due to increased free radical concentration, since the ESR signal intensity (amplitude) is proportional to the concentration of radicals in the sample (Grech et al 1996).

FIGURE 4.0: TYPICAL PRE AND POST EXERCISE ESR SPECTRA OF PBN ADDUCT IN HUMAN SERUM.

PRE-EXERCISE

POST-EXERCISE



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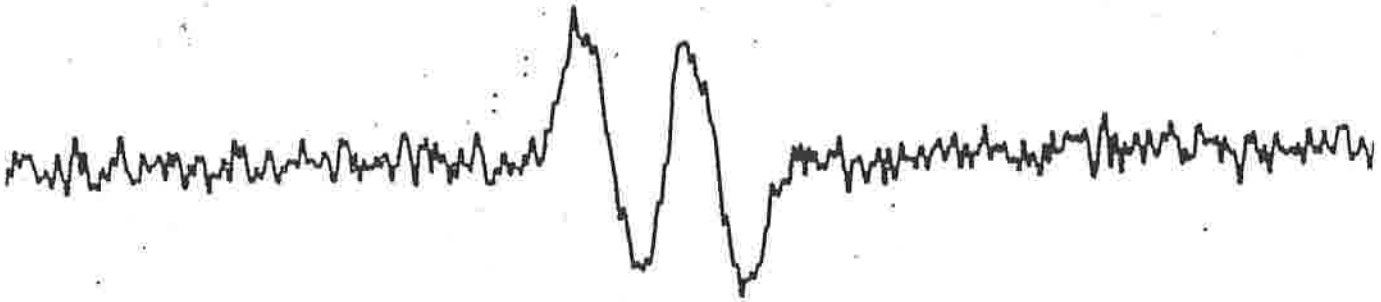
0.45 mT

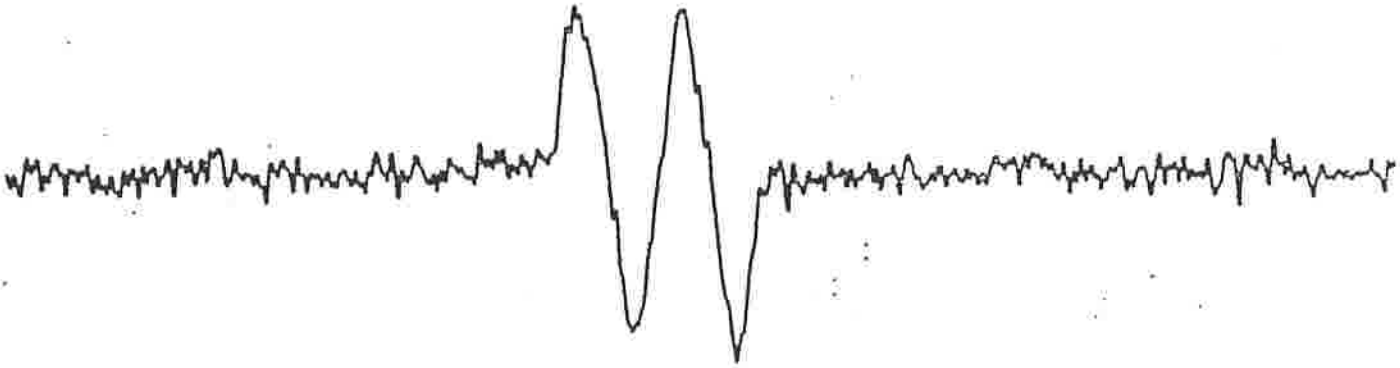
Hyperfine coupling constants are: a_N = 1.371 mT, and a_H = 0.194 mT.

FIGURE 4.1: TYPICAL ESR SPECTRA OF ASCORBYL RADICAL IN HUMAN PLASMA.

PRE-EXERCISE



POST-EXERCISE



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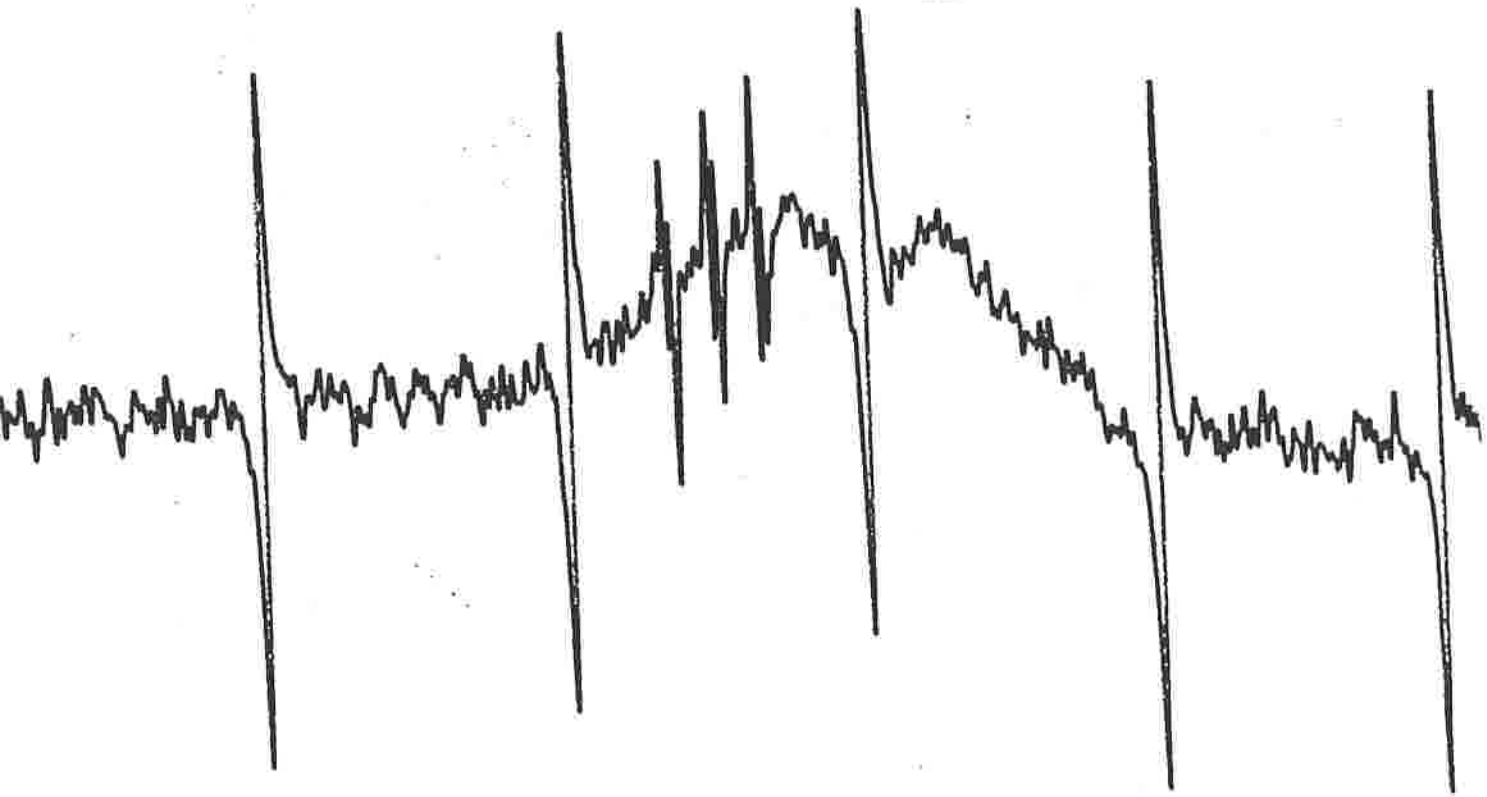
0.129 mT

Hyperfine coupling constant: a_H: 0.183 mT.

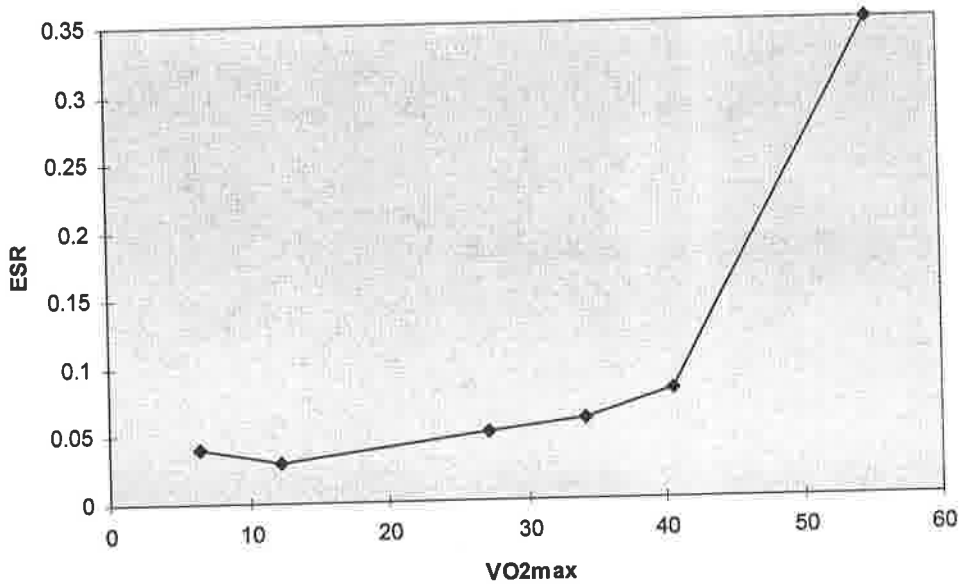
FIGURE 4.2: REPRESENTATIVE POST-EXERCISE ESR SPECTRA WITH MANGANESE MARKER.



g-value: 2.006.

Figure 4.2 shows the ESR spectra of the PBN adduct in human serum post-exercise. The spectrometer is equipped with a calibrated manganese marker which is inserted into the cavity. The marker consists of manganese-oxide yielding a characteristic six line ESR spectrum due to the presence of Mn^{2+}. The 4th line has a known g-value which acts as a standard to measure other g-values. From this information the g-value of the signal was determined and found to be 2.006. This value is consistent with a radical species found in human blood reported by Grech et al (1996).

FIGURE 4.3: FREE RADICAL PRODUCTION DURING MAXIMAL AEROBIC EXERCISE.



ESR = arbitrary units, VO_2 = ml.kg.min^{-1}

Figure 4.3 shows the progressive increase in the concentration of the PBN adduct and hence free radicals, in the sera of one subject, during a maximal oxygen uptake test. These novel data illustrate the existence of a "threshold" of increase in free radical-mediated oxidative-stress during exercise of this type. This is potentially clinically important since this would allow an exercise intensity to be

prescribed where the benefits of exercise would accrue without the deleterious effects of increased oxidant concentration.

FIGURE 4.4: THE RELATIONSHIP BETWEEN OXYGEN UPTAKE AND THE ESR SIGNAL INTENSITY OVER TIME DURING A VO_{2MAX} TEST.

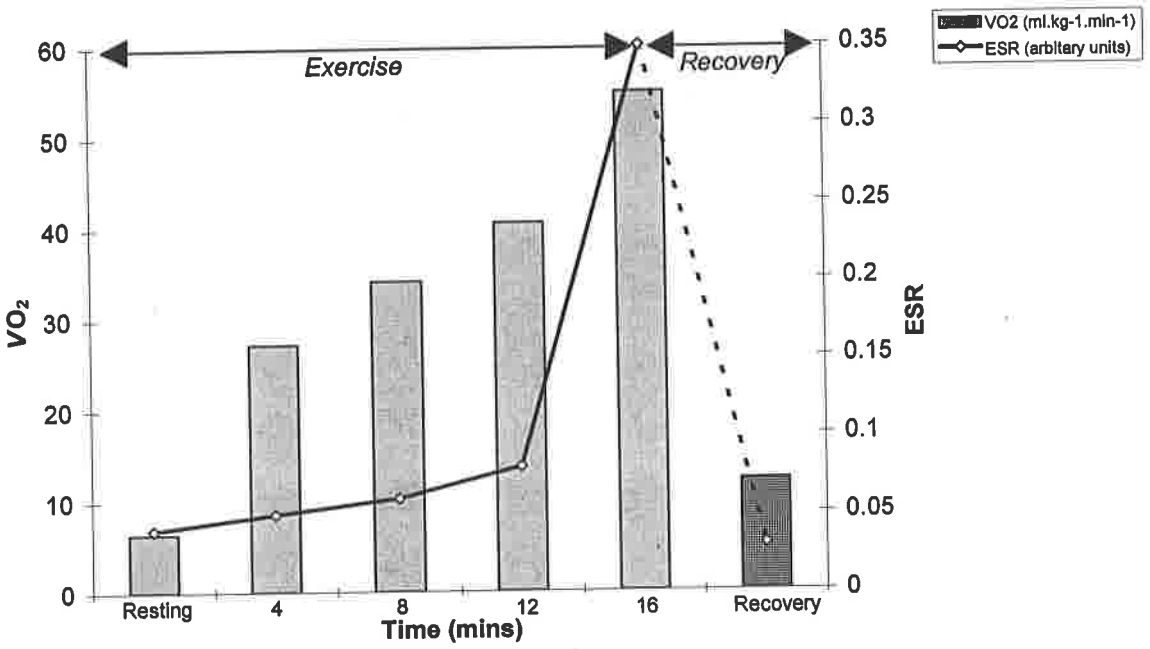


Figure 4.4 demonstrates the relationship between oxygen uptake over time and the "threshold" of increased free radical production.

Table 4.2 summarises the main results of this study and clearly show that maximal aerobic exercise induces measurable changes in the concentration of free radicals as measured by ESR, and free radical-mediated lipid peroxidation (LH and MDA) in the human venous circulation. In addition to these data, this type of exercise also results in statistically significantly increased concentrations of bacterial endotoxin in the plasma of these apparently healthy subjects.

FIGURE 4.6: THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON SERUM PBN ADDUCT CONCENTRATION.

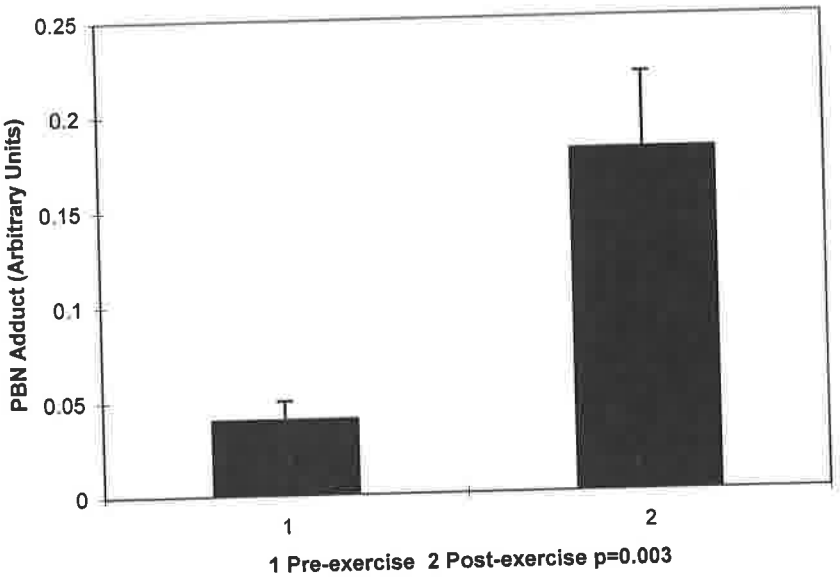


Figure 4.6 shows the effect of exhaustive aerobic exercise on the concentration of the PBN adduct in human serum. It can be seen that exhaustive aerobic exercise results in statistically significant increases in the concentration of the PBN adduct and hence free radicals in human serum.

FIGURE 4.7: THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON PLASMA ASCORBYL RADICAL CONCENTRATION.

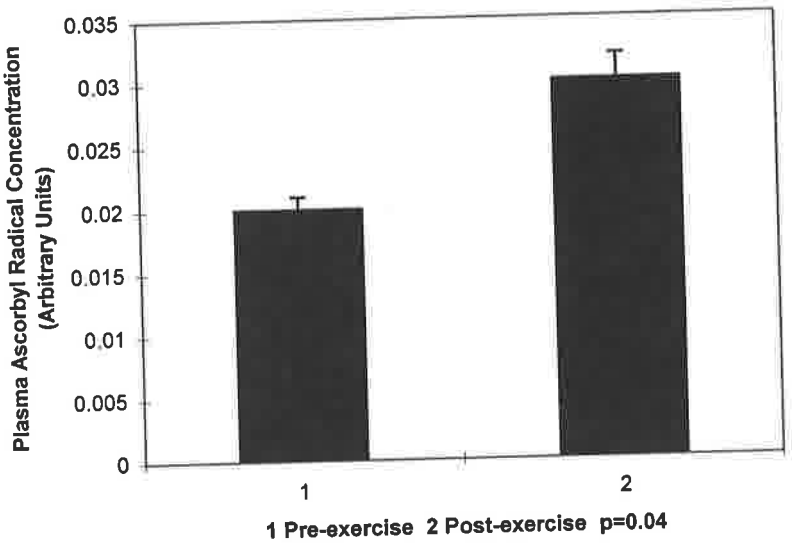


Figure 4.7 shows the effect of exhaustive aerobic exercise on the concentration of the ascorbyl radical in human plasma. Exhaustive aerobic exercise resulted in a statistically significant increase in the concentration of the ascorbyl radical. Suggesting that exercise of this nature induces oxidative stress as indicated by the increased concentration of the ascorbyl radical.

FIGURE 4.8: THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON PLASMA MALONDIALDEHYDE CONCENTRATION.

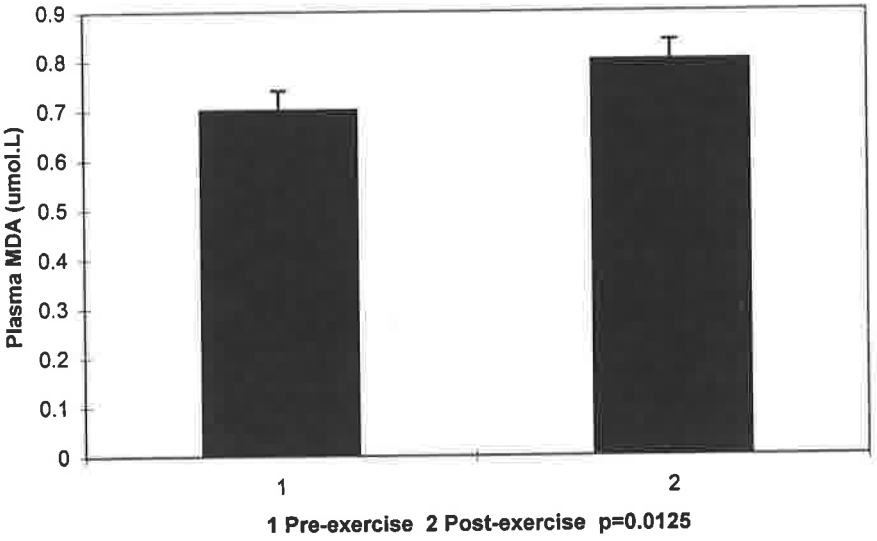
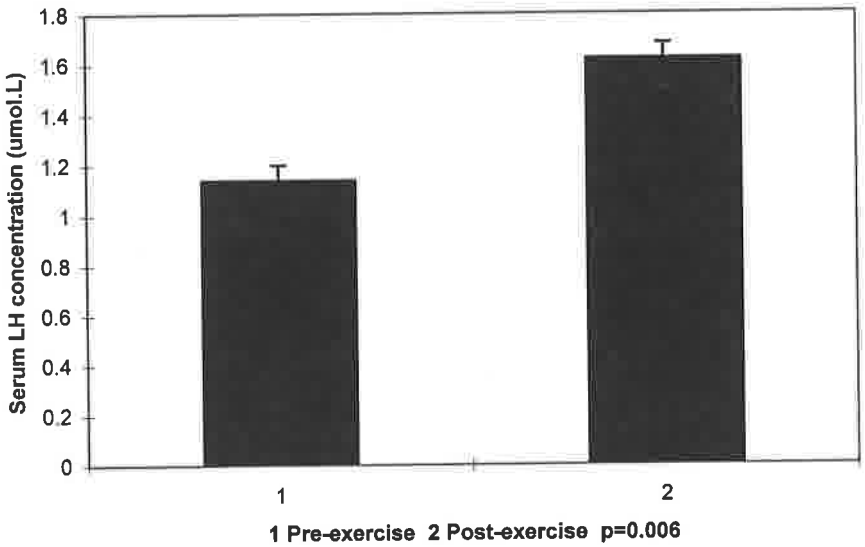


FIGURE 4.9: THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON SERUM LIPID HYDROPEROXIDE CONCENTRATION.



Figures: 4.8 and 4.9 show the effect of exhaustive aerobic exercise on free radical-mediated lipid peroxidation products in human blood. It can be seen that this type of exercise induces significant increases in the concentration of MDA and

LH in the human venous circulation. The implication is therefore, that exercise of this type causes increased free radical production which in turn cause lipid peroxidation, thus allowing the measurement of increased concentrations of these products. One interpretation of this is that increased oxygen flux is central to the observed increase in these products of oxidative damage.

FIGURE 4.10: THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON PLASMA ENDOTOXIN CONCENTRATION.

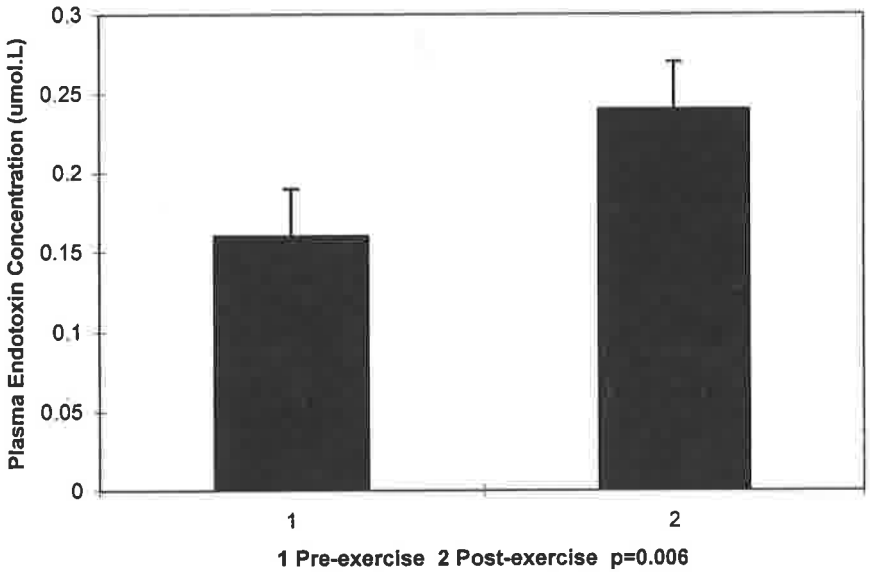


Figure 4.10 shows the effect of exhaustive aerobic exercise on the concentration of bacterial endotoxin (lipopolysaccharide) in human plasma. It can be seen that this type of exercise induces statistically significant increases in endotoxin concentration. These data would appear to support the hypothesis that exercise-induced redistribution of blood away from the gut to the working muscles leaves the gut in a state of relative ischaemia. On cessation of exercise, reperfusion occurs leading to oxygen-centred radical formation possibly by a xanthine-oxidase mechanism which causes oxidative damage to the gut mucosa allowing translocation of endotoxin into the circulation. However, it is suggested that a

gradual leakage of endotoxin into the circulation occurs during exercise as the gut becomes progressively ischaemic due to increasing exercise intensity. This results in significant increases in plasma endotoxin post-exercise as observed.

TABLE 4.3: EFFECT OF EXHAUSTIVE EXERCISE ON BLOOD ANTIOXIDANT STATUS.

	Pre-exercise	**Post-exercise**
TAC ($\mu mol.L^{-1}$ Trolox eq)	518 ± 38.4	574 ± 51.9 **ns**
Ascorbic acid ($\mu mol.L^{-1}$)	18.44 ± 5.25	21.71 ± 5.12 **ns**
α-tocopherol ($\mu mol.L^{-1}$)	14.58 ± 3.42	14.82 ± 3.53 **ns**
β-carotene ($\mu mol.L^{-1}$)	0.08 ± 0.02	0.09 ± 0.02 **ns**
α-carotene ($\mu mol.L^{-1}$)	0.01 ± 0.03	0.01 ± 0.03 **ns**
Lycopene ($\mu mol.L^{-1}$)	0.27 ± 0.10	0.27 ± 0.09 **ns**
Retinol ($\mu mol.L^{-1}$)	1.37 ± 0.36	1.31 ± 0.30 **ns**

NB: Results are expressed as mean ± SEM, p-value for Wilcoxon signed rank test for all results $p>0.05$. Total antioxidant capacity (TAC), ns, not significant.

Table 4.3 shows the effect of exhaustive aerobic exercise on blood antioxidant status. It can be seen that this type of exercise does not induce any significant changes in blood antioxidant status.

4.2.6: INTER-ASSAY CORRELATIONS.

Positive correlations were seen between lipid hydroperoxides (LH) and ESR pre-exercise ($r=0.80$, $p=0.009$) and post exercise ($r=0.71$, $p=0.02$), which suggests that they are products of the same origin ie. increased free radical production. However, a positive correlation exists between malondialdehyde (MDA) and ESR pre-exercise ($r=0.73$, $p=0.02$) but not post-exercise ($r=0.32$, $p=0.38$), the lack of correlation post-exercise may reflect inadequacies in the clearance of MDA. A

very weak inverse relationship appears to exist between post-exercise ESR signal intensity and total antioxidant capacity (r= -0.15, p=0.79), although this is not significant and may therefore be due to chance. Positive correlations were also seen between maximal oxygen uptake and post-exercise MDA and LH (r= 0.50, p= 0.006, r= 0.30, p= 0.34) respectively. These correlations may well be important since they implicate oxygen uptake in the production of free radical-mediated products of lipid peroxidation. Maximal oxygen uptake, as expected, was positively correlated with maximum heart rate (r=0.72, p=0.008).

Importantly a significant positive correlation exists between ESR post-exercise and maximal oxygen uptake (r= 0.76, p=0.004), and ESR post-exercise and maximum heart rate (r= 0.48, p= 0.005). In this author's opinion, this is very important new information since it strongly implicates oxygen uptake as a potential cause of the observed significant increase in the concentration of the PBN adduct and ascorbyl radical post-exercise.

4.3 Discussion.

The effect of maximal aerobic exercise on the ESR signal in human blood.

The purpose of the present study was to determine the effect of exercise eliciting maximal oxygen uptake on free radical production *in vivo*, as measured by ESR spectroscopy. Furthermore to demonstrate the effect of strenuous aerobic exercise on other supporting indices of oxidative damage including lipid peroxidation and endotoxin production. Results of this study indicate a parallel stimulation by exercise in all five main assays (post vs. pre exercise, mean ± SEM, p value for Wilcoxon signed rank test, $p<0.05$). There was a significant post-exercise increase in the normalised ESR signal intensity measured in arbitrary units. Figure 4.0 shows typical ESR spectra of the PBN adduct and illustrates an

increase post-exercise. ESR conditions were identical before and after exercise the only exception being alterations in gain, which in general was lower post-exercise. The hyperfine coupling constants were a_N = 1.371 mT and a_H = 0.194 mT. The species have therefore been tentatively identified as being either carbon-centred or oxygen-centred radicals using previously published tables (Buettner 1987).

The coupling constants seen in the present study compare favourably with literature reports of PBN adducts in human blood drawn from a variety of circumstances known to result in increased free radical concentration. For example, Tortolani et al (1993) reported the existence of alkoxyl (a_N = 1.36 mT, a_H = 0.19 mT) and carbon-centred (a_N = 1.41 mT, a_H = 0.42 mT) radicals in the blood of patients undergoing elective cardioplegia. The authors suggest that the alkoxyl radicals may arise as a fragmentation product of tissue-derived hydroperoxides following reaction with extracellular catalytic iron, and that the carbon-centred radicals are formed via β-scission of the alkoxyl radicals (Tortolani et al 1993). This is supported by Grech et al (1996) who detected radicals in the blood of patients following primary percutaneous transluminal coronary angioplasty with nitrogen coupling constants ranging from a_N = 1.47 to 1.54 mT. The authors suggest that the radicals are oxygen-centred alkoxyl radicals or carbon-centred radicals formed via secondary reaction of oxygen-derived radicals with membrane lipid components. However, due to poor signal-to-noise ratio caused by the necessary use of high modulation amplitude the a_H values are unreported (Grech et al 1996). This is further supported by evidence from Coghlan et al (1991) who reported coupling constants (a_N = 1.45 to 1.58 mT and a_H = 0.15 to 0.16 mT consistent with a PBN trapped carbon-centred radical in toluene from patients undergoing coronary angioplasty. Furthermore Coghlan et al (1991) detected lipid hydroperoxides in the blood samples, which may be due to free radical-mediated lipid peroxidation. The authors conclude

that ESR spin trapping is a feasible technique to measure free radical presence in humans (Coghlan et al 1991). Importantly therefore for the present study, the efficiency of toluene extraction of a PBN adduct from blood has been reported to be 85-90% with no significant artifactual signals, and further that *ex vivo* ESR spin trapping is feasible in blood (Mergner et al 1991).

The cellular origin of the ESR signals observed in the present study is supported by Dickens et al (1991) who detected alkoxyl radicals (a_n = 1.375 mT and a_H = 0.225 mT) in endothelial cells on re-oxygenation. It is suggested therefore that the radicals are secondary species formed as a consequence of primary oxygen-centred radical attack on the cell membrane resulting in lipid peroxidation. This is supported by evidence from Bolli et al (1988) who reported the trapping by PBN of secondary oxygen- and carbon-centred radicals with coupling constants ranging from: a_N = 1.475 mT to 1.500 mT; and a_H = 0.267 mT to 0.279 mT. They suggest that the species are either alkoxyl or alkyl radicals respectively and may be formed by reactions of primary oxygen radicals with membrane lipids (Bolli et al 1988).

The mechanism of production of the PBN adducts observed in the present study however may also involve the univalent reduction of oxygen pathway (which occurs in the mitochondria) and concomitant superoxide production, since it has been shown that PBN adduct production in stunned myocardium is inhibited by superoxide dismutase and catalase (Bolli et al 1989). This would therefore appear to implicate mitochondrial electron transport chain leakage and the "oxygen-flux" hypothesis as the origin of the increased ESR signal seen post-exercise in the present study.

Furthermore Mergner et al (1991) suggest that PBN adducts may be generated from analogues of lipid hydroperoxides. This again implicates primary oxygen-

centred radicals as the cause of the increased products of free radical-mediated lipid peroxidation seen in the present study, since decomposition of lipid hydroperoxides can lead to alkoxyl radical production (Tortolani et al 1993). Importantly the radical species detected in the present study compare favourably to oxygen-centred radical adducts of PBN (a_N = 1.370 mT, a_H = 0.183 mT) detected in hepatic organic extracts as reported by Reinke et al (1991). Due to the numerous potential mechanisms of production of free radicals *in vivo*, it is extremely difficult to determine with any degree of certainty the precise origin of the PBN adduct seen in the present study.

The ESR detection of oxygen-centred radicals is difficult due to high reactivity of for example, the hydroxyl radical with biological molecules (Finkelstein et al 1980). A reaction rate constant of 10^8 to 10^{10} $M.s^{-1}$. has been reported (de Groot 1994). One interpretation of this is that the species are secondary alkoxyl radicals, possibly derived from peroxidation of membrane lipids by primary oxygen-centred radicals.

The high oxygen uptake achieved in this study may represent high oxygen flux through the tissues and concomitant flow of electrons down the mitochondrial electron transport chain, since mitochondria utilize approximately 85-90% of the oxygen intake (Gutteridge and Halliwell 1994). This can result in enhanced electron leakage as a consequence of increased whole body oxygen uptake possibly leading to the formation of primary oxygen-centred radicals such as superoxide (Strain 1995). It is hypothesised that it is these primary radicals that initiate membrane lipid peroxidation leading to the formation of alkoxyl and carbon-centred lipid-derived secondary radicals. It is these radicals that are then detected by ESR and are thus responsible for the increased ESR signal observed post-exercise in this study.

Sommani and Arroyo (1995) have demonstrated the existence of both the ascorbyl radical (a_H = 0.189 mT) and a PBN adduct (a_N = 1.63 mT, a_H = 0.35 mT) in cardiac tissue of exercise trained rats, which compare favourably to those seen in the present study. The authors have attributed the PBN adducts to a lipid peroxidation by-product (Sommani and Arroyo 1995). However, they suggest that the presence of the ascorbyl radical in the heart tissue of exercise trained rats is indicative of protection due to the formation of the ascorbate free radical. This is at variance with Buettner and Jurkiewicz (1993) who suggest that the presence of the ascorbyl radical is indicative of oxidative stress. This is supported by Sharma and Buettner (1993) who observed increases in the ascorbyl radical (a_H = 0.18 mT) in human plasma subjected to continuous oxidative stress.

The conclusion drawn by Sommani and Arroyo (1995) seems somewhat unwise since in rested control rats in their study there was no ascorbyl radical signal. The oxidation-reduction of ascorbic acid involves the generation of the intermediate ascorbyl radical and thus logically it could be assumed that increased amounts of the ascorbyl radical are indicative of consumption of the antioxidant vitamin C in response to an oxidative challenge.

The significant increase in free radical-mediated lipid peroxidation products seen in the present study supports the hypothesis that the origin of the PBN adduct involves peroxidation of membrane lipids and are possibly due to lipid hydroperoxide production. However since the type of exercise performed in this study resulted in significant increases in oxygen uptake, then ultimately mitochondrial electron transport chain leakage may be indirectly responsible for the large post-exercise increase in ESR signal intensity. It is important to note that providing more oxygen for mitochondria does not result in increased ROS formation *per se* but in enhanced leakage of electrons from the electron transport

chain and hence greater formation of superoxide (Gutteridge and Halliwell 1994).

In such a complex biochemical system as human blood it is virtually impossible to categorically determine the origin of the observed signal, since there are numerous recognised pathways for free radical production *in vivo*. Inference can only be made as to where the species originated by observed changes in supporting assays and the physiological response to exercise. It is also a severe limitation in the field of free radical biochemistry that it is difficult to correlate the formation of specific free radicals with cellular injury (Rosen and Halpern 1990). A discussion of some of the many possible *in vivo* sources responsible for the changes reported in this study will now be presented, with reference to the scientific literature to support these novel results.

It is suggested that the origin of the observed increase in the ESR signal intensity of the PBN adduct is leakage of electrons from the mitochondrial electron transport chain causing superoxide and other reactive species to be formed. Mitochondria have been described as the most important source of superoxide *in vivo* (Halliwell and Gutteridge 1989). The rationale for this is that aerobic exercise causes large increases in whole body oxygen uptake which results in a vastly increased flow of electrons down the respiratory chain of the mitochondria. The 15% of electrons that leak to form superoxide (Sawyer 1988), is amplified by at least 10 to 15 fold, thus providing an environment conducive to increased free radical formation. Production of superoxide may also occur secondary to mitochondrial O_2 reduction (Zweier et al 1989).

There is substantial evidence that mitochondria are a source of potentially toxic oxygen-derived free radicals (Ambrosio et al 1993, Turrens et al 1991, Loschen et al 1974, Zhang et al 1990). In addition to this enhanced rate of electron leakage,

mitochondria yield substantial amounts of hydrogen peroxide and superoxide at rates that are dependent upon mitochondrial oxygen utilisation, the site of this is thought to be ubiquinone, where ubiquinone reacts in its reduced form with molecular oxygen to produce hydrogen peroxide (Forman and Boveris 1982). Thus the higher the oxygen utilisation by mitochondria, the greater the production of ROS by mitochondria.

Oxygen uptake by active skeletal muscle may increase 40-fold above resting values (Aw et al 1986), while oxygen flux in individual muscle fibres may increase 100-200 fold (Strain 1995), importantly muscle fibres are themselves richly endowed with mitochondria (Tortora and Grabowski 1996). The significance of this in relation to exercise is that the mechanism of aerobic energy transduction and the release of partially reduced oxygen intermediates share the same biochemical system: the mitochondrial electron transport chain.

Mitochondria themselves are composed of an inner and outer membrane consisting of approximately 80% protein and 20% lipid and, 50% lipid and 50% protein respectively (Devlin 1997). The unsaturated lipids in the mitochondrial membrane are themselves suceptible to oxidant attack as are the proteins leading to formation of lipid peroxidation products and protein carbonyls respectively.

Unbound coenzyme-Q (2,3-dimethoxy-5-methyl-6-multipenyl-1,4-benzoquinone) is a lipid-soluble quinone that can carry electrons rapidly through the inner mitochondrial membrane to cytochrome reductase. Coenzyme-Q cycles between the reduced ubiquinol and the oxidized ubiquinone as it transfers electrons from complex I to complex III. It has been suggested that all H_2O_2 is produced by dismutation from $O_2^{\cdot-}$ rather than direct two-electron transfer to O_2 (Forman and Boveris 1982). Significantly coenzyme-Q itself is thought to be the main source of H_2O_2 in the mitochondrial inner membrane although reduced coenzyme-Q may

also act as an antioxidant (Boveris et al 1976). The ESR detection of the ubiquinone radical following exhaustive exercise has been previously reported and may therefore have contributed indirectly to the post-exercise increase in the ESR signal intensity observed in this study via production of hydrogen peroxide (see section 2.1.1).

As electrons pass from carrier to carrier, protons are pumped from the matrix of the mitochondria to the intermembrane space, the imbalance in proton concentration establishes a proton gradient across the membrane which is used for ATP synthesis. Under resting states the synthesis of ATP is tightly coupled to electron flow. This coupling reduces the flow of electrons when ADP becomes limiting and increases it when ADP becomes plentiful, as during strenuous exercise. This tight regulation is known as respiratory control. Tight coupling prevents unnecessary oxygen consumption when the supply of ATP is adequate. This may be considered an indication of oxygen toxicity since aerobic exercise requires large amounts of oxygen, then activity in the respiratory chain increases to meet the demand for ATP.

Exhaustive aerobic exercise has been shown to result in loss of respiratory control, increased lipid peroxidation, decreased coupling of energy transduction and oxygen uptake, and increases in the post-exercise ESR signal intensity of active skeletal muscle tissue (Davies et al 1982). The signal was attributed to ubiquinone, thus implicating mitochondria as the origin of the ESR signal.

Given the previous discussion the mechanism of oxidant-mediated cell injury in the present study probably involves superoxide formed by one electron reduction of oxygen via electron transport chain leakage followed by a concomitant increase in hydrogen peroxide via spontaneous dismutation, and dismutation by superoxide dismutase. Hydrogen peroxide is known to be able to

readily diffuse across membranes and exert damage on critical cellular targets such as DNA, mediate loss of intracellular ATP, oxidize NADPH and form the hydroxyl radical (Boveris et al 1972, Hyslop et al 1988, Dupuy et al 1991).

In addition to the above mechanism, hyperoxia (which the lungs especially would be exposed to in this exercise test) has been shown to increase hydrogen peroxide generation in lung mitochondria (Turrens et al 1982). Furthermore mitochondria generate hydrogen peroxide when the partial pressure of oxygen increases (Boveris and Chance 1973), as may theoretically occur in the lungs during a maximal oxygen uptake test.

Mitochondria are high oxygen consuming organelles due to their central role in cellular ATP supply thus the mitochondrial respiratory function is crucial to all aspects of cell metabolism. This is important since one aspect of training adaptation is mitochondrial hypertrophy and hyperplasia (Sanders-Williams 1986), leading to increased potential for ROS generation. It may be that the improvement in antioxidant defences observed with training compensate for this phenomenon.

Importantly the ascorbate free radical [$A^{\cdot-}$] was detected by ESR in plasma pre and post-exercise (see figure 4.1). There is a statistically significant increase in the concentration of the ascorbyl radical as detected by ESR pre vs. post-exercise (0.02 ± 0.001 vs. 0.03 ± 0.002 arbitrary units). This increase may in part reflect the non-significant post-exercise increase seen in plasma ascorbic acid concentration (18.44 ± 5.25 to 21.71 ± 5.12 μmol/L pre vs. post-exercise). It has however recently been suggested that changes in ascorbyl radical concentration be used as a sensitive index of oxidative stress, since the ascorbyl radical is a resonance-stabilized tricarbonyl species that is readily formed from the one-electron oxidation of the ascorbate anion [AH^-] (Buettner and Jurkiewicz 1993).

The low reduction potential of $A^{\cdot-}$ / AH^- couple, $E^{\circ\prime}$ = +282 mV means that virtually every oxidizing radical that arises *in vivo* will bring about the one electron oxidation of AH^-, forming $A^{\cdot-}$. Thus the steady state concentration of $A^{\cdot-}$ may be used as a sensitive measure of oxidative stress *in vivo* (Buettner and Jurkiewicz 1993).

It is postulated therefore that the post-exercise increase in ascorbyl radical was not due to any increase in plasma ascorbate concentration (since there was no significant increase), but a physiological response to exercise-induced oxidative stress. Thus reflecting oxidation of ascorbate to dehydroascorbic acid with the formation of the intermediate ascorbyl radical.

This is supported by studies demonstrating an increase in the concentration of the ascorbyl radical in isolated hepatocytes undergoing oxidative stress, and is proposed to be an early index of oxidative damage (Tomasi et al 1989). Therefore the significant post-exercise increase in the concentration of the ascorbyl radical is indicative of oxidative stress occurring in the blood caused directly by maximal aerobic exercise eliciting maximal whole body oxygen uptake.

The transition from rest to exercise is accompanied by dramatic changes in both the rate of fuel utilization by muscle and distribution of blood flow, exercise also stimulates the utilization of glycogen and glycerides stored in muscle (Saltin et al 1986). The rate of glycogen utilisation and the relative importance of this substrate in fuel metabolism is dependent upon the intensity and duration of the exercise, thus with increasing work intensity carbohydrate oxidation is predominant, which has been confirmed by studies measuring Respiratory Exchange Ratio (RER) (Saltin et al 1986).

Results from the present study indicate a mean RER of 1.23 ± 0.03 at termination of test suggesting carbohydrate oxidation may predominate. Within minutes of the onset of exercise blood flow to the working muscles increases several fold, delivering large amounts of oxygen and substrate after being diverted from other regions notably the kidney and gut. The substrate is primarily glucose, derived from increased rates of hepatic glucose production and local muscle glycogen stores, as well as free fatty acids from adipose tissue lipolysis.

In addition to these changes, hormonal responses to exercise lead to increased levels of circulating adrenaline and nor-adrenaline rising during exercise (Bjorkman 1986). This is potentially significant in this study since auto-oxidation of adrenaline has been shown to increase superoxide and hydrogen peroxide production (Loschen et al 1974). Thus another possible source *in vivo* may contribute to the post-exercise increase in the ESR signal intensity observed in the present study.

Furthermore, whole body dynamic exercise such as cycling results in large groups of muscle working simultaneously. Blood flow to the working muscles is increased resulting in ischaemia in other tissues notably the gut and kidney, which may also contribute to the increased ESR signal intensity observed post-exercise in this study. Rapid alkoxyl radical formation on reoxygenation of hypoxic endothelial cells has been reported by Dickens et al (1991), which may implicate lipid peroxide decomposition as a potential source of ROS. This may be important since in this study lipid hydroperoxides increased significantly post-exercise (1.15 ± 0.07 pre-exercise to 1.63 ± 0.29 μmol.L, p=0.006 post-exercise).

It is also important to note the possible role skeletal muscle plays in the generation of ROS following strenuous aerobic exercise. Muscle contraction in general, may be concentric, eccentric or isometric in nature (McComas 1996).

During contraction opposing actin filaments are propelled toward each other and slide along the intervening myosin filament: the sliding filament theory first postulated by Huxley (McComas 1996). Projections from the myosin filaments known as cross bridges lie in six rows along the myosin filament and momentarily attach themselves to the actin filaments and propel them to new positions. Each cross bridge acts as an independent force generator and the force developed is dependent upon the number of simultaneous interactions between the cross bridges and the actin filaments (McComas 1996). Importantly cycling has eccentric as well as concentric components which may contribute to the generation of ROS.

The neurochemical signal for muscular contraction brings about an increase in the concentration of Ca^{2+} ions in the vicinity of the actin and myosin filaments. Thus Ca^{2+} acts as an intermediary between the action potential and the contractile apparatus. The cellular mechanism by which the Ca^{2+} concentration is increased is known as excitation-contraction coupling and occurs in two stages:

1. Depolarization of the T-tubules.
2. Diffusion of Ca^{2+} ions from the sarcoplasmic reticulum to the myofilaments.

A significant aspect of the role of Ca^{2+} in muscle contraction is its association with free radical generation (Jackson 1996). It has been proposed that muscle damage associated with free radical increase following uphill and downhill running is more dependent upon mechanical stress rather than the metabolic cost of the activity (Eston et al 1996).

However, during strenuous exercise the demand for ATP may outstrip supply which may be manifested by an increase in ADP and Pi. Furthermore insufficient respiration in the mitochondria could feasibly lead to lower levels of ATP which

would affect Ca^{2+} extrusion from the cytoplasm via ATP-dependent Ca^{2+} pumps in the sarcolemma, mitochondria and sarcoplasmic reticulum, this is unlikely to occur in eccentric exercise since energy utilisation is low (Armstrong 1990). The importance of external Ca^{2+} influx, and Ca^{2+} activation of phospholipase A_2 leading to fatty acid release from membranes, in free radical-mediated muscle damage has been established (Jackson 1990, Jackson et al 1984, Jones et al 1984).

A role for oxygen in the pathogenesis of exercise-induced muscle damage is possible since the generation of available energy supplies is dependent upon a vastly increased oxygen flux during exercise (Jackson 1992), which is the case in the present study. The role of free radicals in exercise-induced muscle damage is also supported by studies demonstrating the protective effect of the antioxidant vitamin E (Jackson 1987).

Furthermore oxygen and pro-oxidants can generate excitability in lipid bi-layers causing them to become selectively permeable to Ca^{2+} ions, whilst antioxidants may modify the ion selective properties of the bilayer (Lebedev and Levitsky 1989). Therefore oxygen, peroxides from membrane lipids and free radicals may play an important role in the regulation of Ca^{2+} transport through cellular membranes via modification of the lipid hydrophobic barrier (Lebedev and Levitsky 1989). Although the link between free radical mediated muscle damage and Ca^{2+} is difficult to confirm, Ca^{2+} mediated release of membrane fatty acids may cause increased free radical production and lipid peroxidation.

These "free" fatty acids are more susceptible to free radical-mediated lipid peroxidation since once released from the membrane absence of lipid-soluble antioxidants such as vitamin E, no longer protect them from peroxidation by free radicals, in addition to this the cytoplasm may be a major site of free radical

production during normal metabolism (Jackson and Edwards 1986). Thus another possible mechanism of origin exists for the increase in the ESR signal intensity post-exercise reported in this thesis.

Figure 4.3 provides novel ESR information regarding the production of free radicals during exercise and illustrates a dramatic increase in the ESR signal intensity of the PBN adduct. The point at which the graph ceases to be linear is approximately 41 $ml.kg.min^{-1}$ of oxygen uptake which for the subject corresponds to 74% of maximal oxygen uptake. Although no comparable ESR data exists, previous studies examining lipid peroxidation at different exercise intensities have alluded to a threshold of increased free radical production during exercise at between 60% and 77% of VO_{2max} (Sen et al 1994, Kanter et al 1993). The novel ESR data presented in this thesis is thus in agreement with the indirect evidence reported in the literature.

The data presented in figure 4.3 suggest that this threshold corresponds to approximately 74% of VO_{2max}. However despite this and other data the threshold for exercise-induced oxidative stress remains to be discovered since a large sample number would be required.

The ESR signal amplitude returned to near baseline levels at 4 minutes post-exercise reflecting the decrease in oxygen consumption on cessation of work. However oxygen consumption at this point was slightly greater than the baseline pre-exercise values. This reflects the well known increase in resting oxygen consumption and metabolic rate that occurs following strenuous exercise of this nature and is related to the accumulated oxygen debt (Astrand and Rodahl 1986).

The ESR signals of the PBN adduct probably belong to secondary radical species since the half life of primary oxygen radicals would generally be too short to

permit trapping. The increase in lipid peroxidation post-exercise and the positive correlation between ESR and lipid peroxidation supports this conclusion and implicates lipid peroxidation as a possible mechanism. In addition to this a decreased intensity in the high field lines of the ESR spectrum is indicative of spin-inhibition, one interpretation of this is that the spin trap is carrying a large bio-molecule such as a long chain polyunsaturated fatty acid of membrane origin. Rosen and Finkelstein (1985) have stated that in the presence of unsaturated membrane lipids allylic hydrogen abstraction by hydroxyl radical results in the formation of a lipid radical. This can react with molecular oxygen to yield the corresponding lipid hydroperoxyl radical. Thus the production of superoxide can result in the generation of at least four distinct radical species: hydroxyl; superoxide anion; lipid radical; lipid hydroperoxyl radical (Rosen and Finkelstein 1985).

It has been shown that *in vitro* oxidation of erythrocyte membranes proceed by a chain mechanism with a long kinetic chain length (Niki et al 1991). The inverse relationship, although non-significant, between antioxidant status and ESR signal intensity post-exercise may implicate increased free radical production brought about by exercise. Although it should be stressed that the relationship between exercise, antioxidant status and free radical production must be viewed with caution due to the lack of statistical significance.

Perhaps however, the most important evidence presented in this thesis to support the hypothesis of the role of increased oxygen uptake in the generation of increased free radical production is the statistically significant strong positive correlation ($r = 0.76$, $p=0.004$) between the post-exercise ESR signal intensity and maximal oxygen uptake. This suggests a link between the post-exercise increase in the ESR signal intensity of both the PBN adduct and the ascorbyl radical, and increases in products of free radical-mediated lipid peroxidation. This is further

supported by the statistically significant positive correlation between ESR post-exercise and maximum heart rate ($r= 0.48$, $p= 0.005$). Thus strongly implicating oxygen flux and by definition the mitochondrial electron transport chain as the origin of the observed increase in ESR signal intensity.

In addition to these data a manganese marker of known g-value was attached to the spectrometer and a g-value for the signal obtained (see figure 4.2). The g-value of the ESR signal is 2.006 which is compatible with a radical of biologic origin as stated previously. Furthermore the hyperfine splitting constants are compatible with the spin trapping by PBN of either a carbon-centred species or an alkoxyl radical either of which could be formed by secondary reactions of primary oxygen radical reaction with membrane lipid components (Garlick et al 1987, Tortolani et al 1993, Bolli et al 1988).

The comparatively small a_H coupling constant of the PBN adduct is indicative of a bulky attached group (Janzen and Blackburn 1968). One interpretation of this as stated previously, is that the attached group may be a large bio-molecule of lipid origin that results in spin inhibition. The identification and possible origin of the PBN species is further explored in chapter seven: *in vitro* studies.

THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON LIPID PEROXIDATION IN HUMAN VENOUS CIRCULATION.

Following exercise there was a significant increase in lipid peroxidation as assessed by both MDA from 0.70 to 0.80 $\mu mol.L^{-1}$ and LH from 1.15 ± 0.07 to 1.63 ± 0.29 $\mu mol.L^{-1}$. This is in agreement with several reports in the literature (see section 2.2). The origin of the increased concentration of lipid peroxides in the blood may have several sources including; catecholamines released during intensive exercise, liberation of iron from ferritin (Yagi 1992), and possibly the

cellular membranes themselves. Lipid peroxides may originate from primary oxygen-centred radical attack on cell membrane PUFA resulting in secondary lipid- derived and peroxyl radical formation which propagate the peroxidation resulting in accumulation of LH and MDA. Thus the possibility exists that the increase in lipid peroxidation seen in the present study implicates the cell membrane in the mechanism of formation of LH and MDA.

Importantly the correlation between the assays of lipid peroxidation and ESR suggests that the assays are measuring the same phenomenon. Furthermore ESR spin trapping evidence has demonstrated that the primary radical involved in membrane lipid peroxidation is the ·OH radical (Lai and Piette 1977).

The production of $O_2^{\cdot-}$ *in vivo* resulting in peroxidation to membrane polyunsaturated lipids causes alterations in cell membrane fluidity, it has been shown for example that $O_2^{\cdot-}$ causes human myelin to change from an ordered to a disordered liquid crystalline state (Chia et al 1983). This loss of membrane fluidity results in leakage of intracellular hydrolytic lysosomal enzymes. It is worth noting that even brief episodes of lipid peroxidation results in long lasting membrane damage (Flohe et al 1978). This may explain the increased levels of MDA observed several days after exercise reported by Meydani et al (1993).

Possibly the most damaging aspect of lipid peroxidation is its self-perpetuating nature and the fact that lipid peroxidation reaction products may diffuse away from the site of origin to produce damage at a new site (Benedetti et al 1979). Increased susceptibility to lipid peroxidation as measured by the thiobarbituric reactive substances (TBARS) assay has been observed in the erythrocytes of sports people (Anuradha et al 1995). This is indicative of oxidative damage in a potentially detrimental location *in vivo* since the erythrocyte is well endowed

with iron that can catalyse hydroxyl radical formation from H_2O_2 via Fenton chemistry.

Moderate exercise has been reported to result in a 62% and 90% increase in the MDA content of fast-twitch oxidative and fast-twitch glycolytic muscle respectively (Alessio and Cutler 1990). The same group reported a more dramatic increase with high-intensity exercise of 167% and 157% respectively. Thus this exercise test resulted in known products of oxidative damage being measured in increased amounts in human serum. The increased concentration of lipid hydroperoxides and malondialdehyde observed in the present study is therefore in agreement with published reports. Furthermore the increased concentration of lipid peroxidation products seen following exercise supports the novel ESR data, suggesting that they are measuring similar phenomena.

THE EFFECT OF STRENUOUS AEROBIC EXERCISE ON PLASMA ENDOTOXIN LEVELS.

Strenuous exercise triggers an inflammatory response which bears some resemblance to those events occurring in sepsis (Brock-Utne et al 1988, Bosenberg et al 1988). Thus one of the main features of strenuous muscular exercise is its ability to induce an inflammatory immune response (Schaefer et al 1987). During this response arachidonic acid is liberated from cell membranes by phospholipase A_2 resulting in activation of the arachidonic acid cascade; cytokines are produced; leukocytes are released and activated; complement is activated and free radical production is increased (Camus et al 1993). Despite this information the mechanism of exercise-induced systemic endotoxaemia seems poorly understood.

During exercise, under the influence of the autonomic nervous system, blood is shunted away from internal organs such as the gut, towards active skeletal

muscle. The gut is thus in a state of relative ischaemia until reperfusion occurs on cessation of exercise. Ischaemia-reperfusion has been shown in other settings to result in a marked increase in both intra and extra-cellular oxygen free radical concentration and subsequent tissue damage (Baker et al 1988, Baker and Kalyanaraman 1989).

In the present study exhaustive aerobic exercise resulted in a statistically significant increase in plasma endotoxin (0.16 ± 0.03 to 0.24 ± 0.06 $EU.ml^{-1}$, $p=0.001$). The origin of this increase is unknown. It is however hypothesised that translocation of endotoxin across the gut mucosa into the bloodstream following oxidant damage during exercise-induced gut ischaemia and reperfusion is responsible for the increase, ie. that exercise-induced systemic endotoxaemia is a free radical mediated process. This is separate and distinct from the acute phase immune response often seen following strenuous exercise particularly if muscle damage is invoked, although raised plasma endotoxin may contribute to the fever and cytokine response reported following strenuous exercise (Tidball 1995, Northoff et al 1994).

Importantly there is some evidence that strenuous muscular exercise triggers an inflammatory response which is similar to those occurring in sepsis, and therefore exercise and sepsis may share the common pathway of endotoxin release in blood (Camus et al 1994). The factor triggering this release could well be oxidant-mediated damage to the gut mucosa during exercise-induced gut ischaemia allowing bacterial endotoxin to translocate into the bloodstream. Muscle damage allowing diffusion of cellular debris through the damaged sarcolemma thus attracting leukocytes and activating the complement cascade as a possible triggering mechanism has been proposed (Camus et al 1994).

It is known that exposure to endotoxin can prime neutrophils to release large amounts of superoxide at levels that result in death to mice (Yoshikawa 1990), and that circulating neutrophils produce ROS following exhaustive exercise (Suzuki et al 1996), which has been confirmed by ESR and spin trapping techniques (Britigan 1987). Furthermore it has been demonstrated by Stark et al (1988) that endotoxin administration to mice results in noticeable increases in the ESR signal of the ascorbyl radical. It is worth speculating that the observed increase in the plasma ascorbyl radical concentration seen in the present study post-exercise is influenced by plasma endotoxin concentration.

The elucidation of the precise mechanism would be of clinical importance, and may well involve conversion of xanthine dehydrogenase to xanthine oxidase with the production of the superoxide anion (Sjodin et al 1990). It may also involve stimulation of neutrophils to release ROS.

The precise mechanism however is still as yet unknown since it appears, to this authors knowledge, only three studies have measured exercise-induced endotoxin release in humans which were following ultra-endurance events (Bosenberg et al 1988, Brock-Utne et al 1988, Moore et al 1995). Further studies are warranted to attempt to elucidate the precise mechanisms regarding free radical mediation of systemic endotoxaemia and its biological significance. Evidence regarding this phenomenon is currently lacking particularly with regard to short-term acute exercise.

THE EFFECT OF MAXIMAL AEROBIC EXERCISE ON BLOOD ANTIOXIDANT STATUS.

Plasma antioxidant status appeared to be unaffected by exhaustive aerobic exercise. There was no significant change in any of the antioxidant parameters measured in this study including: TAC; ascorbic acid; α-tocopherol; β-carotene; α

-carotene; lycopene; and retinol. The fact that statistical significance was not achieved may be due to sample numbers and/or large standard deviation. However a trend to increase plasma antioxidant parameters post-exercise was observed in the presnt study. The increase in antioxidant parameters reported in the literature may reflect a host response to the increased levels of circulating free radicals brought about by the strenuous nature of the exercise test. Workers have however reported a significant increase in plasma antioxidant status post-exercise in supplemented individuals (see section 2.3.3).

4.4 CONCLUSION.

Regular physical exercise and sports performance are known to be beneficial to health and this is evidenced by the lower level of cardiovascular disease in physically-active individuals, however, the evidence presented in the present study corroborates work in animals that free radical production increases during exercise in a variety of tissues including blood. The toxicity of excess oxygen may therefore be due to increased formation of $O_2^{\cdot-}$ formed by faster electron leakage or from autoxidations of molecules such as catecholamines, which overwhelms the antioxidant defence mechanisms (Aruoma 1994). This may cause increases in the ESR signal and other indices of oxidative damage including lipid peroxidation and endotoxin translocation.

It seems clear that exercise increases free radical production and lipid peroxidation and this may be detrimental to health. Endurance or aerobic training may reduce lipid peroxidation by increasing superoxide dismutase and catalase activity; however data are conflicting. Aerobic cycle training has been shown to result in increased whole body maximal oxygen consumption although muscle superoxide dismutase, catalase and glutathione peroxidase activities

were not altered by training (Tiidus et al 1996). Results indicate that moderate aerobic training typically performed by regularly exercising humans did not positively alter endogenous antioxidant status. This suggests that aerobic training increases the capacity for flux through the citric acid cycle without improving the ability of the body to cope with the free radicals generated by the enhanced mitochondrial electron flux (Tiidus et al 1996).

It has been stated that the unequivocal demonstration of increased free radical activity in complex biological tissues is difficult and is usually only accepted if a variety of indicators provide supportive evidence (Jackson 1996).

Furthermore this evidence can be in the form of indirect indicators of free radical activity such as products of lipid peroxidation or prevention by administration of antioxidants (Jackson 1996).

The present study has sought to do the former while study three (chapter 6) seeks to do the latter. Therefore it can be cautiously concluded that exercise causes increased free radical concentration as demonstrated by statistically significant increases in the ESR parameters of the PBN adduct and the ascorbyl radical. Additionally this is supported by significantly increased levels of free radical-mediated lipid peroxidation products (LH and MDA) and also raised concentration of blood lipopolysaccharide.

The scientific research community has been encouraged to identify those for whom involvement in exercise would be deleterious to health due to cardiac considerations (Shephard 1984). It is therefore suggested by this author, that this be extended to consider the risks to health of long-term exercise in relation to the as yet unknown cumulative effects of increased free radical production. Jenkins (1988) has indicated that never in history has so large a cross-section of humans

engaged in such high oxygen consumption doses over so long a portion of their lives. The suggestion that anything consumed in sufficiently high doses may have the potential to harm has, in this author's opinion, never been more appropriately applied than to the study of exercise-induced oxidative stress !

Chapter Five

Study 2:

THE INFLUENCE OF ANAEROBIC EXERCISE ON FREE RADICAL PRODUCTION

5.0 INTRODUCTION.

Ashton et al (in press) have reported a statistically significant increase in the ESR spectra of the PBN adduct and ascorbyl radical in human blood following maximal aerobic exercise. Additionally they have shown statistically significant increases in supporting assay of lipid peroxidation following the same exercise test. The authors have suggested that the mechansim of increase implicates increased whole body oxygen uptake and enhanced leakage from the mitochondrial electron transport chain leading to formation of secondary radicals possibly derived from peroxidation of membrane PUFA.

A test of this suggested mechanism is that anaerobic exercise where whole body oxygen uptake is not significantly increased should not lead to increases in the post-exercise ESR signal. Therefore, the aim of the present study was to determine the effect of anaerobic metabolism on *in vivo* free radical production, since the majority of published reports have examined free radical production exclusively as a function of aerobic exercise. To this authors knowledge, there currently exists no data on the effects of anaerobic exercise on the ESR signal intensity in the human venous circulation. Also there appears to be no data on the effect of the Wingate anaerobic exercise test on the other indirect indices of oxidative stress such as lipid peroxidation.

The logic for concentrating on aerobic metabolism has been that as oxygen uptake or "flux" increases, then leakage of electrons from the electron transport chain also increases, and hence a threshold of oxidative stress is exceeded. This result has been demonstrated in study 1. In order to test the role of oxygen in free radical production during exercise, an anaerobic study was performed. It is hypothesised that if electron transport chain leakage and/or partially reduced oxygen intermediates are the origin of the increased ESR signal and lipid

peroxidation observed in study 1, then anaerobic exercise where maximal whole body oxygen uptake is not achieved during the exercise test, should result in little or no increase in the ESR signal intensity (unless other mechanisms are active). The present study was therefore designed to examine the effects of anaerobic exercise, which elicits a large increase in lactic acid concentration and relies predominantly on non-oxygen dependent pathways to provide energy for muscular work (Astrand and Rodahl 1986), on exercise-induced free radical production.

Few studies have examined predominantly anaerobic exercise in relation to free radical production. It has however been demonstrated that high intensity sprinting exercise results in increased levels of lipid peroxidation in rat skeletal muscle (Alessio et al 1988). The study reported a 90% and 160% increase following a 1 minute sprint at 45 m/min, in slow twitch and fast twitch muscle respectively. The muscle fibres examined were soleus (red slow twitch) and red and white vastus (fast twitch) (n=8).

The results suggest that a mechanism exists *in vivo* for the production of these derivatives of oxidative damage during all out sprinting. An important point is that the greatest increase was observed in fast twitch fibres suggesting an inadequate ability to remove LH and MDA by these fibre types, this may be reflected in the lower concentrations of superoxide dismutase and catalase found in glycolytic fibres, as indicated by the relationship between oxygen uptake and markers of oxidative damage (Jenkins et al 1984). The authors speculate that the increased concentration of these products occurs as a result of the imbalance between production and clearance of both LH and MDA, due to exercise-induced changes.

It is pertinent also to note that the greatest increase was observed in fast twitch glycolytic fibres. The significance being that anaerobic metabolism is predominant in this muscle type. This in turn could render the muscle ischaemic during intense periods of work such as sprinting, causing significantly increased free radical production on reperfusion by a xanthine oxidase mechanism.

Repetitive static exercise (RSE), a condition of known partial ischaemia / reperfusion, has also been examined in relation to its ability to trigger metabolic and oxidative stress (Sahlin et al 1992). In the above study seven (n = 7), healthy human subjects performed two-legged intermittent knee extension exercises for 10 s on and 10 s off at a target force of 30% maximal voluntary contraction force. The RSE was continued for 80 min (n = 4) or to fatigue (n = 3). Four of the subjects also performed dynamic exercise at an intensity of 60% VO_{2max} for the same period. MDA remained below the detection limit in all conditions, while blood lactate remained low in all subjects. The rationale for the possibility of increased free radical production may be that during muscle contraction blood flow is restricted due to increased intramuscular pressure which results in hyperaemia during the relaxation phase. Thus ischaemic tissues may experience increased oxidant concentration on reperfusion via a xanthine oxidase mechanism (McCord 1985).

The above exercise studies are at variance with each other although a key point is that RSE was a static exercise protocol and thus oxygen uptake would not be expected to rise rapidly, and this was shown to be so, whereas sprinting is a dynamic exercise and could theoretically involve increased VO_2 uptake.

A further problem arises when comparing these studies in that it is difficult to extrapolate results obtained in rats to humans. The present study seeks to determine the effect of supra-maximal anaerobic exercise on the ESR signal

intensity in human venous blood and to determine if oxidative damage is induced by maximal sprinting.

5.1 METHODOLOGY.

STUDY DESIGN.

Seven (n = 7) healthy male subjects volunteered for the study. They consisted primarily of a cross-section of the student population of UWIC. All were non-smokers and subjects who took vitamin supplements were excluded. Consent and ethical approval was as previously described. The tests were carried out at the Human Performance Laboratory, UWIC, under the same environmental conditions as study 1.

EXERCISE PROTOCOL AND BLOOD SAMPLING.

The exercise tests were performed on a calibrated cycle egometer (Monark 824ε). All blood samples were drawn from an ante-cubital vein using a vacutainer system (Becton-Dickinson Ltd. Oxford, UK) as previously described. The resting, pre-exercise sample was obtained before the warm-up on the ergometer while having been seated in a chair for 5 minutes. The post-exercise blood sample was taken immediately following the test with minimum delay. Although a short delay of 5-10 s was inevitable experimentally. The vacutainers contained the spin trap PBN as previously detailed. Duplicate blood samples were collected for assay of lipid peroxidation and antioxidant status, and treated as described in study 1. In addition to this whole blood lactate was measured pre and post-exercise using a venous blood sample and the Analox blood lactate analyser.

THE WINGATE ANAEROBIC EXERCISE TEST.

A standard anaerobic exercise test (Wingate test) was performed in order to test the effect of anaerobic metabolism on free radical production. The Wingate test is a valid and reliable assessment of anaerobic performance (BASES 1988) and involves a 30 s supra-maximal sprint against resistance calculated relative to bodyweight. The resistance is calculated using the formula of 75g/kg bodyweight and is applied after initial inertia and unloaded frictional resistance is overcome (Medbo and Tabata 1989). This test was chosen since it is a well established method of assessing anaerobic metabolism and power.

PROTOCOL FOR THE WINGATE ANEROBIC EXERCISE TEST.

1. The ergometer seat and handlebar setting was adjusted according to the positions previously recorded.
2. The subject warmed-up by cycling for 5 minutes, at 60 rpm with 60 W (1.0 kg) resistance on the flywheel.
3. The subject dismounted and recovered for 2 minutes.
4. The subject was then taken through a gentle stretching regime by the researcher for a period of 2 minutes. This consisted of; touching toes repeated 5 times, hamstring stretch repeated 5 times, quadriceps stretch repeated 5 times and calf stretch repeated 5 times.
5. The subject was required to cycle maximally for 30 s at a resistance calculated by the formula, 75 g /kg bodyweight (BASES 1988).
6. The researcher held up the weight pan so that no resistance was felt, while the subject cycled to 60 rpm.
7. On reaching 60 rpm, the researcher counted down 3-2-1-GO.
8. Immediately the pan was released the subject simultaneously sprinted maximally for 30 s. The time was monitored by stopwatch.

9. Immediately at 30 s the subject was removed from the ergometer and a blood sample obtained.

NB: Toe clips were used during the test and the subjects were required to remain seated throughout the test. Heart rate was continually recorded during the test by means of a portable heart rate telemetry device (Polar Sport Tester, Polar Ltd. Kenilworth, UK).

STATISTICAL METHODS.

Statistical analysis was identical to that in study ie. non-parametric analysis.

5.2 RESULTS OF STUDY 2: ANAEROBIC EXERCISE AND FREE RADICAL PRODUCTION.

TABLE 5.0: ANTHROPOMETRIC AND NUTRITIONAL DATA.

Height (cm)	Body mass (kg)	BMI	Age (years)	Energy (kcals)	Fat (grams)	Protein (grams)	CHO (grams)
179.07 ± 2.61	79.29 ± 5.48	23.9 ± 1.8	25.0 ± 7.0	3704 ± 417	163.1 ± 10.2	146.0 ± 19.9	441.2 84.6

NB: Results are expressed as mean, ± SEM. BMI, body mass index (kg/m^2); CHO, carbohydrate.

Table 5.0 shows the anthropometric and nutritional data of the sample. It can be seen that the subjects are non-obese, but with a higher fat and energy intake when compared to study 1.

FIGURE 5.0: TYPICAL PRE AND POST ANAEROBIC-EXERCISE ESR SPECTRA OF PBN ADDUCT IN HUMAN SERUM.

PRE-EXERCISE

POST-EXERCISE

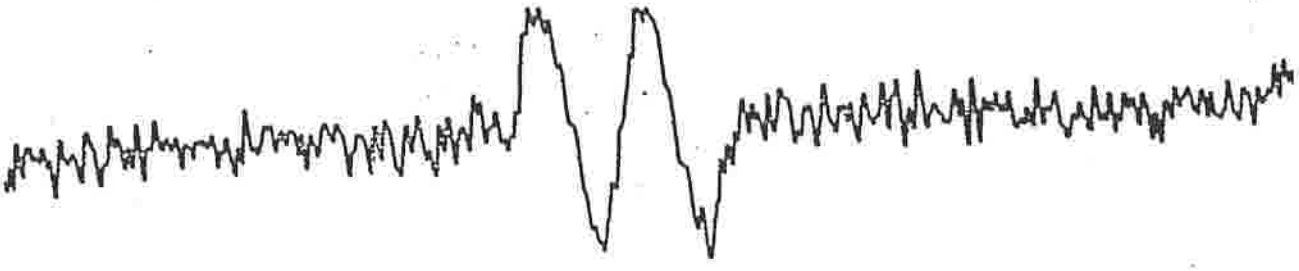
--------->

0.45 MT

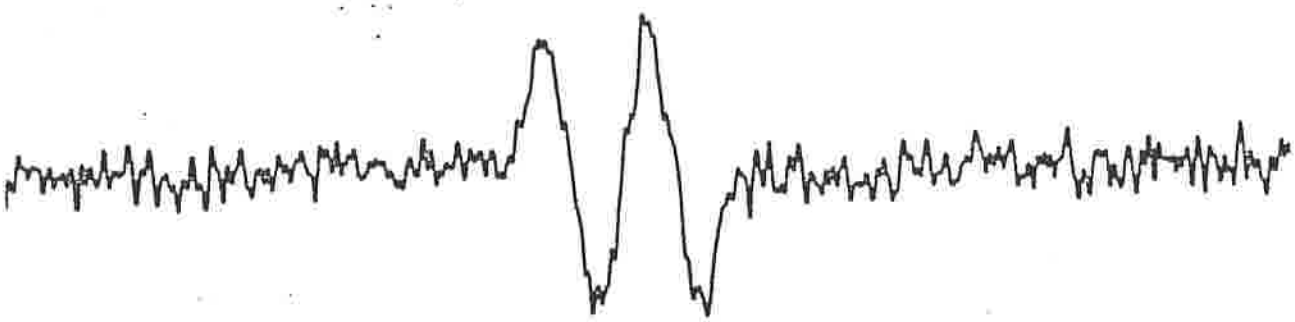
Hyperfine coupling constants: a_N = 1.350mT, and a_H = 0.190 mT.

FIGURE 5.1: TYPICAL PRE AND POST ANAEROBIC EXERCISE ESR SPECTRA OF THE ASCORBYL RADICAL IN HUMAN PLASMA.

PRE-EXERCISE



POST-EXERCISE



----->

0.129 MT

Hyperfine splitting constant: a_H = 0.183 mT.

TABLE 5.1: THE EFFECT OF SUPRA-MAXIMAL ANAEROBIC EXERCISE ON OXIDATIVE STRESS PARAMETERS AND LACTATE CONCENTRATION IN HUMAN BLOOD.

	PRE-EXERCISE	POST-EXERCISE
Heart rate (beats.min^{-1})	66.0 ± 1.79	122 ± 4.68, **p=0.03**
PBN adduct (Arbitrary units)	0.02 ± 0.01	0.08 ± 0.03 **ns**
Ascorbyl radical (Arbitrary units)	0.01 ± 0.004	0.02 ± 0.006 **ns**
MDA (μmol.L^{-1})	0.74 ± 0.08	0.94 ± 0.11, **p= 0.01**
LH (μmol.L^{-1})	1.57 ± 0.62	1.73 ± 0.65 **ns**
Lactate (mmol.L^{-1})	2.30 ± 0.12	11.34 ± 1.14, **p= 0.02**

Results are mean ± SEM; MDA, malondialdehyde; LH, lipid hydroperoxides; p-value for Wilcoxon signed rank matched pairs test.

Table 5.1 shows the effect of strenuous anaerobic exercise on various blood parameters and heart rate. It can be seen that heart rate increases above resting, pre-exercise levels following performance of the Wingate anaerobic exercise test. This may be important since it indicates an aerobic response to an established anaerobic exercise test. The table shows that strenuous anaerobic did not cause any significant increase in the concentration of either the PBN adduct or the ascorbyl radical in human plasma. Which therefore supports the hypothesis that tissue oxygen flux is important in generation of increased oxidant concentration following aerobic exercise. This is supported by a lack of increase in blood lipid hydroperoxide concentration. However, the significant post-exercise increase in plasma MDA concentration is at variance with the hypothesis, although this may reflect imbalance in the clearance of MDA from the blood rather than increased production, since MDA is formed by breakdown of lipid hydroperoxides as

shown in the biochemical model (see figure 5.4). As expected, blood lactic acid concentration significantly increased above resting levels following the Wingate test. This indicates predominantly anaerobic metabolism.

TABLE 5.2: THE EFFECT OF ANAEROBIC EXERCISE ON BLOOD ANTIOXIDANT STATUS.

	Pre-exercise	**Post-exercise**
TAC ($\mu mol.L^{-1}$)	1162.50 ± 52.9	1207.50 ± 61.4 **ns**
Ascorbic acid ($\mu mol.L^{-1}$)	46.34 ± 7.98	53.18 ± 8.37 **ns**
α-tocopherol ($\mu mol.L^{-1}$)	18.40 ± 3.24	19.23 ± 3.97 **ns**
β-carotene ($\mu mol.L^{-1}$)	0.14 ± 0.03	0.10 ± 0.05 **ns**
α-carotene ($\mu mol.L^{-1}$)	0.05 ± 0.02	0.08 ± 0.06 **ns**
Lycopene ($\mu mol.L^{-1}$)	0.49 ± 0.06	0.46 ± 0.08 **ns**
Retinol ($\mu mol.L^{-1}$)	2.57 ± 0.14	2.63 ± 0.25 **ns**

NB: Results are mean, ± SEM; p value for Wilcoxon matched pairs signed rank test; TAC, total antioxidant capacity.

Table 5.2 demonstrates that strenuous anaerobic exercise results in no significant change in blood antioxidant status. This may be due to sample numbers as opposed to the effect of exercise, although comparably there was no significant increase in study one with n=12 subjects. Therefore it is fair to say that exhaustive anaerobic exercise results in no change in a variety of blood antioxidant parameters.

TABLE 5.3: COMPARISON OF RESULTS BETWEEN STUDIES ONE (AEROBIC) AND TWO (ANAEROBIC).

	STUDY 1		STUDY 2	
	Pre-exercise	**Post-exercise**	**Pre-exercise**	**Post-exercise**
PBN adducts (Arbitrary units)	0.04 ± 0.01	0.18 ± 0.04 **p= 0.003**	0.02 ± 0.01	0.08 ± 0.03 **ns**
Ascorbyl radical (Arbitrary units)	0.02 ± 0.001	0.03 ± 0.002 **p= 0.04**	0.01 ± 0.004	0.02 ± 0.006 **ns**
MDA ($\mu mol.L^{-1}$)	0.70 ± 0.05	0.80 ± 0.04 **p= 0.0125**	0.74 ± 0.08	0.94 ± 0.12 **p= 0.01**
LH ($\mu mol.L^{-1}$)	1.15 ± 0.07	1.63 ± 0.29 **p= 0.006**	1.57 ± 0.62	1.73 ± 0.65 **ns**
TAC ($\mu mol.L^{-1}$)	518 ± 38.4	574 ± 51.9 **ns**	1162.50 ± 52.9	1207.50 ± 61.4 **ns**
Ascorbic acid ($\mu mol.L^{-1}$)	18.44 ± 5.25	21.71 ± 5.12 **ns**	46.34 ± 7.98	53.18 ± 8.37 **ns**

NB: PBN, phenylbutyl-tert-nitrone; MDA, malondialdehyde; LH, lipid hydroperoxides; TAC, total antioxidant capacity; ns, not significant, p value for Wilcoxon matched pairs test.

Table 5.3 compares the results of aerobic vs anaerobic exercise. Results indicate statistically significant increases in all parameters of oxidative stress following strenuous aerobic exercise, whilst anaerobic exercise resulted in no change (with the exception of MDA) in levels of oxidative stress, when measured by ESR or lipid peroxidation. Plasma ascorbic acid concentration remained unchanged in

either of the studies post-exercise. Importantly however, it should be noted that plasma ascorbic acid concentration and hence plasma TAC was greater than reported in study one which may act as a confounding factor when attempting to make inter-study comparisons.

INTER-ASSAY CORRELATIONS.

There was no significant correlation between post-exercise ESR and post-exercise lactate concentration ($r= -0.19$, $p= 0.67$). This was replicated between MDA and LH and lactate concentration. Thus it can be said that in general there was no relationship between any index of oxidative damage and lactate production. This is at variance with the work of Coghlan et al (1991) who reported a positive correlation ($p<0.025$) between coronary sinus lactate levels and detection of PBN adduct levels. The authors suggest that this correlation is indicative of a relationship between the severity of ischaemic insult and free radical formation, which occurs only during reperfusion. It may be assumed that the reason for the discrepancy between the present study and the work of Coghlan et al (1991) is that in the present study exercise-induced ischaemia does not occur or that during dynamic exercise of this nature oxygen flux is central to the generation of free radicals.

This is supported by a lack of correlation between antioxidant status and lactate production, since if a relationship exists between lactic acid and free radical production an inverse relationship between lactic acid concentration and antioxidant status may be anticipated. The lack of such relationships implies that free radicals were not generated to any significant degree, or the plasma was able to mount an effective antioxidant defence. It is suggested that the latter may well be the case due to the noticeably higher plasma TAC levels seen in the present study when compared to study 1. There was no significant correlation between

dietary fat intake and pre-exercise MDA and LH (r= 0.97, 0.87 respectively, p= 0.15), indicating there was no relationship between dietary fat intake and any index of lipid peroxidation in the present study.

5.3 Discussion.

The effect of intense anaerobic exercise on the ESR signal intensity in human blood.

The purpose of the present study was to determine the effect of intense anaerobic exercise on the ESR signal intensity of the PBN adduct and ascorbyl radical in human plasma. Figure 5.0 illustrates a typical ESR spetcra from the present study demonstrating a lack of increase following anaerobic exercise. Mean results show that performance of an established, maximal anaerobic exercise test (Wingate test) results in no significant increase in the ESR signal intensity of both the PBN adduct and ascorbyl radical (figures 5.0 and 5.1 respectively) post-exercise in human plasma.

It is suggested that the lack of increase is due to the fact that whole body oxygen flux is not increased by such extreme amounts as during aerobic exercise. This is evidenced by the minimal increase in heart rate, which has a linear relationship to oxygen uptake (Astrand and Rodahl 1986), observed during this test. Consequently other energy pathways predominate in supplying substrate to the working muscles most notably the anaerobic alactate (creatine phosphate) and anaerobic lactate (glycolytic) pathways. This lack of dependence on the oxidative phosphorylation of ADP coupled to the mitochondrial electron transport chain, is crucial during very intense exercise such as the Wingate test. The significantly large increase in blood lactate concentration (2.30 vs. 11.34 $mmol.L^{-1}$, p= 0.02) is evidence of dependence on the anaerobic energy pathways, and possible skeletal

muscle ischaemia. Lactate accumulation in the blood is the result of an imbalance between production and removal, and blood lactate may be thought of as representative of muscle lactate with higher values occurring in muscle (Foster et al 1995). Lactate is not in itself a metabolic end point but may be converted to pyruvate which feeds into the citric acid cycle, or may diffuse into the blood from the muscle and be transported to the liver for use in gluconeogenesis. The anaerobic energy pathways described below provide ATP during intense exercise such as maximal sprinting.

Figure 5.2 shown below demonstrates the potential for estimating the anaerobic provision of ATP in skeletal muscle, however repeated muscle biopsies are required for such measurements. Anaerobic energy production may be considered essential for the maintenance of high-intensity exercise when the demand for ATP is greater than can be provided aerobically.

Figure 5.2: Energy pathways during intense anaerobic exercise.

$$ADP + PCr + H^+ \xrightarrow{cpk} ATP + Cr$$

$$Glycogen + 3ADP + 3Pi \Leftrightarrow 3ATP + 2Lactate^- + 2H^+$$

$$2ADP \xrightarrow{ak} ATP + AMP$$

$$AMP + H^+ \xrightarrow{\text{amp deaminase}} IMP + NH_4$$

Where;

cpk, creatine phosphate kinase (Spriet 1995)

ak, adenylate kinase

amp, adenosine monophospahte.

At the onset of high-intensity exercise, anaerobically-derived ATP provides 80% to 90% of the total ATP required since oxygen is in short supply, thus the anaerobic contribution to ATP production during 30 s of intense exercise is 80% : 20% in favour of anaerobic metabolism (Spriet 1995). Thus supporting the use of the Wingate test in the testing of the "oxygen-flux" hypothesis in this thesis. During 30 s of intense exercise the PCr store will be depleted, and the glycolytic rate of ATP production will be approximately 50% of the rate during the first 10 s of exercise (Spriet 1995), thus suggesting that any increase in indices of oxidative damage are due to mechanisms other than mitochondrial electron transport chain leakage.

There was no significant increase in the concentration of the ascorbyl radical post-exercise also, the concentration of the PBN adduct did not increase significantly post-exercise. These data therefore support the hypothesis that oxygen flux and concomitant leakage of electrons from the mitochondrial electron transport chain are central to the generation of increased levels of oxidants observed in the blood of healthy subjects following maximal aerobic exercise, which has been reported by Ashton et al (1998).

However, the mean ESR signal of the PBN adducts increased approximately 55% of the post-exercise value recorded in study 1, although as mentioned this was not significant. One explanation for the lack of significance may be that the sample number was too small or that the non-significant increase may be due to chance. It may also however suggest a minor level of exercise-induced oxidative

stress which may prove to be statistically significant if greater numbers were included, there could be several possible explanations for this.

a. AEROBIC CONTRIBUTION TO THE WINGATE TEST.

The contribution of the aerobic energy pathways to the Wingate test has been reported by several groups and results are equivocal. It has been reported that the oxidative contribution to the Wingate test ranges from 18.5% (Kavanagh and Jacobs 1988) to 40% (Medbo and Tabata 1989) and may may even be as high as 44.3% (Stevens and Wilson 1986).

In the present study heart rate increased to approximately 58% of age-predicted maximum heart rate indicating an increase in whole body oxygen flux. It is traditionally assumed that oxygen consumption is approximately 10% below the heart rate value, therefore whole-body oxygen uptake would have increased approximately 48% during the test. This is compatible with the small non-significant increase in the PBN adduct seen in the present study. This supports the possible existence of a threshold of increase in free radical production as suggested in study 1. Furthermore in the present study oxygen uptake only increased approximately 48%, therefore not exceeding the 74% threshold postulated in study 1 and not resulting in increased exercise-induced oxidative stress.

More recently oxygen uptake during the Wingate test has been reported as being approximately 30 to 50 $ml.kg.min^{-1}$ in well-trained sprint and middle distance runners (Granier et al 1995). The authors suggest that the aerobic and anaerobic fitness of the subjects be taken into consideration when assessing the various energy contributions during exercise tests. The present research study did not

control for this and it is therefore a possible criticism of this study. However Granier et al (1995) used well trained athletes in their study, while in the present study subjects comprised a cross-section of student and staff volunteers from the University of Wales. Also the present study was deliberately cross-sectional in order to gain as much information as possible from a small sample size about the effect of supra-maximal anaerobic exercise on free radical production, although sample numbers would limit inferences made. There is however evidence of an aerobic contribution in the performance of this accepted anaerobic exercise test (Wingate test) as shown by increased heart rate. Therefore if the study were to be repeated, this author would recommend that measurement of whole body oxygen uptake be performed and a larger sample used. It is therefore a weakness of the present study that this was not undertaken since the scientific literature provides evidence of an aerobic contribution to an essentially anaerobic exercise test. A corollary to this view however is that the Wingate test is not strictly an anaerobic test ! However, it is recognised that a major feature of the Wingate anaerobic exercise test is that a large proportion of the work done is anaerobic.

It is proposed that an aerobic contribution to the Wingate test is the explanation for the non-significant increase in the ESR signal intensity of the PBN adduct seen in the present study. These data support the hypothesis that aerobic exercise resulting in maximal whole-body oxygen flux results in a significant increase in exercise-induced oxidative stress where oxygen, and thus mitochondrial electron transport chain leakage, play a central role.

b. ACTIVATION OF ADENYLATE KINASE REACTION.

Adenylate kinase catalyses the conversion of 2 moles of ADP to 1 mole of ATP and AMP respectively. The activation of this pathway may lead to increased free radical production by a xanthine oxidase mechanism. Thus in conditions where

ATP is in short supply as during this type of exercise this pathway provides a possible mechanism for the increased ESR signal .

c. XANTHINE OXIDASE.

The xanthine oxidase system has long been proferred as a source of oxygen radicals, the information being largely derived from ischaemia/reperfusion studies (see chapter two). Briefly, activation of calcium dependent proteases leads to the formation of xanthine oxidase from xanthine dehydrogenase, xanthine oxidase uses molecular oxygen as an electron acceptor forming xanthine and superoxide anion by the following mechanism by Jackson (1994):

Figure 5.3: Xanthine oxidase and calcium in the generation of superoxide anion.

ATP

⇓ ⇐ Excess contractile activity

⇓

Hypoxanthine Ca^{2+} accumulation

⇓ ⇓

Ca^{2+} activated protease

Xanthine oxidase ⇐ Xanthine dehydrogenase

Uric acid + $O_2^{\cdot -}$

The formation of the free purine bases hypoxanthine and xanthine terminates with the irreversible oxidation to uric acid by the enzyme xanthine oxidase. It has been demonstrated that molecular oxygen can serve as the electron acceptor

in this reaction resulting in the univalent reduction of O_2 to $O_2^{\cdot-}$ and H_2O_2 (Fridovich 1980). An important aspect of this is that H_2O_2 is itself a moderately strong oxidant and can readily diffuse across cell membranes. It can also produce the hydroxyl radical by the Haber-Weiss reaction. This is potentially important for the present study since by definition anaerobic exercise is arguably ischaemic. This is supported by the fact that the majority of energy produced during the Wingate test is from anaerobic pathways, as indicated by significant increases in blood lactate concentration.

Changes in hypoxanthine levels have been reported in subjects undertaking ischaemic type exercise (Patterson et al 1982, Bothius et al 1988), thus implicating this pathway as a possible mechanism for the observed non-significant increase in the post-exercise ESR signal. However, there is also ESR evidence that the hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine (Britigan et al 1990). The authors suggested that adventitious iron is responsible for the production of the hydroxyl radical, importantly however iron chelation did not affect superoxide production (Britigan et al 1990), which may then produce hydrogen peroxide and secondary radical species such as the hydroxyl radical following interaction with hydrogen peroxide in the presence of transition metal ions.

The implication however for this research study is that there exists *in vivo* a large amount of iron, although this is usually tightly bound to ferritin and transferrin. Exercise-induced damage to muscle and erythrocytes may however allow iron to become available for participation in the Fenton reaction, yielding oxygen radicals.

THE EFFECT OF INTENSE ANAEROBIC EXERCISE ON LIPID PEROXIDATION.

There was no significant increase in the concentration of lipid hydroperoxides (LH), which are the major initial reaction products of oxygen free radical attack on cell membrane PUFA, post-exercise however, the concentration of malondialdehyde (MDA) did rise significantly. The disparity between the lipid peroxidation data may be explained by the pathway of production (figure 5.4: shown below).

Figure 5.4: PATHWAY OF LIPID PEROXIDATION IN HUMAN BLOOD:

Membrane PUFA

$R^{\cdot}$, O_2 uptake ⇓ Peroxidation

Lipid peroxy radical ($ROO^{\cdot}$)

⇓ $H^{\cdot}$ (abstracted from adjacent PUFA, leading to propagation)

Lipid hydroperoxides (ROOH)

Fragmentation

Other peroxide end-products

Cyclic peroxide

⇓

Cyclic endoperoxide

⇓

MDA

Adapted from: Grech et al (1994).

MDA production involves a number of reactions, it is a secondary product formed from the fragmentation of LH, and may not reflect rapidly changing free radical activity (Grech et al 1994). In this model an accumulation of MDA from LH may reflect inadequacies in the clearance of MDA from the circulation rather than increased production. However, an increase in LH would almost certainly lead to an increase in MDA concentration and thus supra-maximal anaerobic exercise results in some degree of lipid peroxidation in human serum.

Significant post-exercise increases in LH were not achieved and the increase when compared to study one is minimal. However it is apparent that the baseline and post-exercise levels are noticeably higher which may reflect the subjects diet in the days leading up to the test. There is a noticeable increase in the mean fat content of the diet in this study compared to study 1. This reflects the heterogeneity of the sample population and may be a possible criticism of the study when attempting to make inter-study comparisons.

The lack of any significant correlation between total dietary fat and lipid peroxidation reported in the present study however does not implicate fat intake in the occurrence of lipid peroxidation in human serum. It has been demonstrated that dietary restriction modulate the extent of cardiac free radical damage and enhances the antioxidant defence system (Kim et al 1996). Thus in accordance with current recommendations a decrease in dietary fat intake could be recommended and may inhibit lipid peroxidation in humans, although evidence to support this is scarce.

THE EFFECT OF INTENSE ANAEROBIC EXERCISE ON BLOOD ANTIOXIDANT STATUS.

There were no significant increases in any aspect of blood antioxidant status following the performance of supra-maximal anaerobic exercise. However, the

mean plasma concentration of TAC and ascorbic acid is higher both pre and post-exercise compared to study 1. Also, the concentration of lipid-soluble antioxidants, noticeably α-tocopherol and β-carotene, are higher when compared to study 1, as is lycopene, which has recently been postulated as being cardio-protective. The increases in the plasma concentrations of the lipid-soluble antioxidants may reflect the observed increase in dietary fat as compared to study 1.

It is therefore feasible that improved blood antioxidant status seen in these subjects has inhibited free radical production induced by the Wingate anaerobic test, and that if a lower blood antioxidant status was present then a statistically significant increase in the post-exercise ESR signal may have been observed. Antioxidant status may therefore act as a confounding factor when attempting to compare studies 1 and 2.

5.4 Conclusion.

In summary, blood lactate measurements are a means of appreciating the equilibrium between the rate of lactate production and removal determined by the relative kinetics of glycolysis, lactate dehydrogenase and perhaps most importantly mitochondrial respiration (Billat 1996). As exercise continues anaerobic glycolysis becomes the dominant energy pathway with the production of large amounts of lactic acid. The lack of correlation between blood lactate concentration and the ESR signal suggests that anaerobic metabolism would not contribute to any increase observed in the ESR signal intensity.

The present study demonstrated that the performance of a maximal anaerobic exercise test does not lead to any significant increase in the post-exercise ESR signal intensity of the PBN adduct or the ascorbyl radical in the blood of healthy

humans. Furthermore intense anaerobic exercise does not lead to any significant increase in the major products of free radical mediated lipid peroxidation (LH), or antioxidant status. This therefore implicates aerobic metabolism as the origin of the non-significant increase in the post-exercise ESR signal intensity. As discussed earlier there may be as much as a 44% contribution to energy production by aerobic metabolism during a Wingate anaerobic exercise test. These data therefore support the hypothesis that increased oxygen flux combined with enhanced rates of mitochondrial electron transport chain leakage are the origin of the increased ESR signal reported in study 1. However, a further point of note is that the short exercise time may also have prevented increases in free radical concentration which thus implicates total work done by the muscle in free radical increase. A confounding issue however is that the large power outputs seen during a Wingate test may indicate increased mechanical stress which may conversely lead to increased free radical damage due to influx of calcium. The mechanism of calcium influx leading to cellular damage has been described by Jackson (1994).

Thus it can be concluded that intense anaerobic exercise, where the majority of work done is anaerobic, does not lead to any significant increase in the post-exercise ESR signal intensity of either the PBN adduct or the ascorbyl radical. An explanation for this is that there appears to exist a threshold of free radical increase corresponding to approximately 74% of VO_{2max}. During the performance of this predominantly anaerobic test this threshold of oxygen uptake was not reached, therefore there were no significant increases in the concentration of free radicals in the blood.

Chapter Six

Study 3:

EXERCISE-INDUCED OXIDATIVE STRESS: THE EFFECT OF ASCORBIC ACID SUPPLEMENTATION

6.1 INTRODUCTION.

It has been reported that that maximal aerobic exercise elicits significant post-exercise increases in the ESR signal intensity, together with lipid peroxidation in the plasma of healthy human subjects (Ashton et al in press). Furthermore that plasma lipopolysaccharide concentration is also significantly elevated following maximal cycle ergometer exercise and that this is a free radical-mediated phenomenon. Study 2 provided cogent evidence of the centrality of whole body oxygen uptake in the generation of increased concentrations of free radicals and concomitant increases in the post-exercise ESR signal intensity. Study 2 therefore provided evidence to refute the null hypothesis that aerobic exercise, eliciting maximal whole body oxygen uptake, does not lead to increased free radical production.

A further test of the hypothesis that maximal aerobic exercise leads to increased free radical production as measured by ESR spectroscopy, indices of lipid peroxidation and plasma endotoxin concentration is that antioxidant intervention should attenuate any free radicals produced by exercise. The evidence to support this would therefore be a lack of increase in any of the parameters previously shown to increase in study 1.

The aim of the present study is to examine the effect of antioxidant pre-treatment on exercise-induced free radical production in healthy humans. An ascorbic acid intervention study will be carried out to examine the effect on oxidative stress parameters following exercise eliciting maximal oxygen uptake and hence maximal tissue oxygen flux. The purpose of this study therefore, is to attempt to attenuate the production of ROS by strenuous aerobic exercise via increasing the plasma concentration of ascorbic acid which should scavenge any ROS produced.

To this author's knowledge, there currently exists no data on the effect of ascorbic acid supplementation on the ESR signal intensity; lipid peroxidation; or endotoxin concentration in the blood of healthy human subjects following the performance of a maximal aerobic exercise (VO_{2max}) test.

The use of molecular oxygen in biological systems proceeds with the release of energy, but without reactive intermediates, such as superoxide anion and hydroxyl radical, being formed providing of course there is no loss of control in or damage to the cell (Hill 1985). Loss of respiratory control has been demonstrated in the mitochondria of rats exercised to exhaustion which was associated with a three-fold increase in the post-exercise ESR signal intensity (Davies et al 1982).

While molecular oxygen is required for the normal metabolic function of aerobic organisms, including humans, the potential for cells to produce reactive oxygen species (ROS) requires careful control of such species, consequently the antioxidant defence mechanisms developed (Thomas and Aust 1989).

It is clearly established that formation of the hydroxyl radical ($^{\cdot}OH$) accounts for much of the damage done to biological systems by increased generation of superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Aruoma and Halliwell 1987). Furthermore, ATP levels decrease, and glycolytic and mitochondrial synthesis are profoundly affected in cells exposed to $O_2^{\cdot-}$, HOCl, and H_2O_2 (Cochrane 1991). Hydrogen peroxide is generated *in vivo* by a number of sources, including: electron transport systems of mitochondria; endoplasmic reticulum and plasma membrane; and peroxisomes (O'Brien 1989), and of course superoxide dismutase. Thus creating the potential for the antioxidant defence mechanisms to be overwhelmed resulting in oxidative stress.

Vitamin C is an essential antioxidant in humans (and guinea pigs), and in sub-clinical ascorbic acid deficiency oxidative damage is evident in most tissues despite adequate concentrations of other antioxidants including: α-tocopherol; glutathione; superoxide dismutase; and catalase, it may also be critically involved in common human degenerative disorders such as atherosclerosis (Nandi et al 1997). ESR evidence suggests that deficiency of ascorbic acid and inefficiency of the ascorbic acid redox system is implicated in cancer progression (Lohmann 1987). While good evidence exists for the role of free radicals in many disease pathologies, it has therefore also led to promising suggestions for therapeutic approaches (Halliwell 1987).

Free radical production is ubiquitous in all aerobic cells even under healthy conditions, and the task of physicians is to prevent catalytic reactions leading to oxidative stress, and to counteract the relative lack of antioxidants which may exacerbate this phenomenon (Hollan 1995). Despite knowledge of the amount required to prevent overt human deficiency, little is currently known regarding the ascorbic acid level required to achieve optimal physiological status (Levine 1986).

There is good scientific rationale that strenuous exercise leads to excess free radical production and the compensatory role that antioxidant nutrients play against the potentially harmful effects of exercise suggests that, an antioxidant nutrient regimen should form an integral part of any exercise programme (Singh 1992). The majority of studies have examined the effect of vitamin E in relation to exercise and oxidative damage since lipid peroxidation appears to have been the most widely studied aspect of exercise-induced free radical production.

To this authors knowledge, there is currently no published ESR evidence on the effect of vitamin C supplementation on exercise-induced oxidative damage in

human blood. Thus the present study seeks to examine the effect of vitamin C supplementation on the ESR signal in human serum and several other indirect supporting indices of oxidative stress during maximal aerobic exercise. Further there appear to be no studies examining the effect of ascorbic acid on endotoxin translocation into the venous circulation following maximal short-term aerobic exercise.

6.2 METHODS.

STUDY DESIGN.

The sample consisted of ten (n = 10) healthy male volunteers. They consisted of a cross-section of students from the University of Wales and were recruited and tested as described in study 1. All were non-smokers and subjects who took vitamin supplements were excluded.

EXERCISE PROTOCOL.

The subjects were required to perform an exhaustive aerobic exercise test eliciting maximum oxygen uptake (VO_{2max}). This is an identical test to the one described in study 1. Detailed descriptions of criteria for cardiovascular measurements during exercise can be found in chapter 3 and in study 1 and reference is made to these.

BLOOD SAMPLING AND BIOCHEMICAL ANALYSIS.

Venous blood was drawn and treated for ESR analysis using a vacutainer system (Becton-Dickinson Ltd.) as previously described. Duplicate blood samples were taken and analysed for an identical range of biochemical parameters as described in study 1 using the methods described in chapter 3.

ASCORBIC ACID SUPPLEMENTATION.

An acute dose of 1 gram of L-ascorbic acid was given orally 2 hours before the subjects performed the VO_{2max} test as 2 x 500 mg non-effervescent tablets (Hoffman-LaRoche Ltd). The ascorbic acid was in the normal state as opposed to a sustained release form. Two hours was chosen in order to allow blood levels of ascorbic acid to increase following the work of Jones (1983). Data on plasma levels of vitamin C in response to a dose is comparatively scarce (Levine et al 1996). However, it has been reported that cells become saturated with vitamin C at doses of 100 mg with plasma being saturated with doses of 1 gram (Levine et al 1996). Tissue ascorbate concentrations are regulated by two mechansims: the kidneys (which conserve body ascorbate via tubular reabsorption and clear excess blood levels > 65 $\mu mol.L^{-1}$), and by saturable dose-dependent intestinal absorption (Jacob 1996). Subjects chewed the tablets thoroughly and swallowed them under supervision. The subjects rinsed their mouths twice with water and swallowed this water to ensure complete ingestion of the ascorbic acid dose.

STATISTICAL METHODS.

These were identical to study 1 and reference is made to them.

6.3 Results of study 3: The effect of vitamin C supplementation on exercise-induced oxidative stress.

Table 6.0: Anthropometric and nutritional data.

Height (cm)	Body mass (kg)	BMI	Age (years)	Energy (kcals)	Fat (grams)	Protein (grams)	CHO (grams)
177.9 ± 1.6	78.6 ± 3.3	25.08 ± 1.6	26.1 ± 5.0	2456 ± 263	90.0 ± 10.7	94.8 ± 11.9	337.9 ± 41.5

NB: Results are expressed as mean, ± SEM. BMI, body mass index (kg/m^2); CHO, carbohydrate.

Table 6.0 shows the subjects height and weight, the BMI indicates that the subjects are non-obese. The subjects nutritional intake is in accordance with current recommendations (DoH 1991).

TABLE 6.1: CARDIOVASCULAR AND HAEMATOLOGICAL DATA.

	VO_2 uptake (ml.kg. min^{-1})	Heart rate (beats. Min^{-1})	Time to exhaustion (min)	RER	Haem-atocrit (% PCV)
Pre-Exercise	7.98 ± 1.21	66 ± 2	NA	0.78 ± 0.03	42.8 ± 1.5
Post-Exercise	47.43 ± 1.95 **p= 0.005**	187.1 ± 3 **p= 0.005**	16.29 ± 1.03	1.21 ± 0.02 **p= 0.005**	43.9 ± 2.4 **ns**

NB: Results are expressed as mean, ± SEM. RER, respiratory exchange ratio; PCV, packed cell colume; ns, not significant; p value for Wilcoxon signed rank matched pairs test.

Table 6.1 shows the cardiovascular and haematological results of the sample. VO_{2max} is similar to the results obtained in study 1, as is maximum heart rate and haematocrit. The time to exhaustion is almost identical compared to study 1, while RER is also very similar when compared to study 1. Thus it is fair to say that this aerobic exercise test eliciting maximal oxygen uptake provided an almost identical physiological challenge and hence, it is assumed oxidative challenge to the exercise test performed by the subjects in study 1.

Figures 6.0 and 6.1 overleaf show typical ESR spectra of the PBN adduct and ascorbyl radical in human plasma. The hyperfine coupling constants are discussed in detail in section 6.4.

FIGURE 6.1: TYPICAL PRE AND POST- EXERCISE ESR SPECTRA OF PBN ADDUCT IN HUMAN SERUM SUPPLEMENTED WITH ASCORBIC ACID.

PRE-EXERCISE

POST-EXERCISE

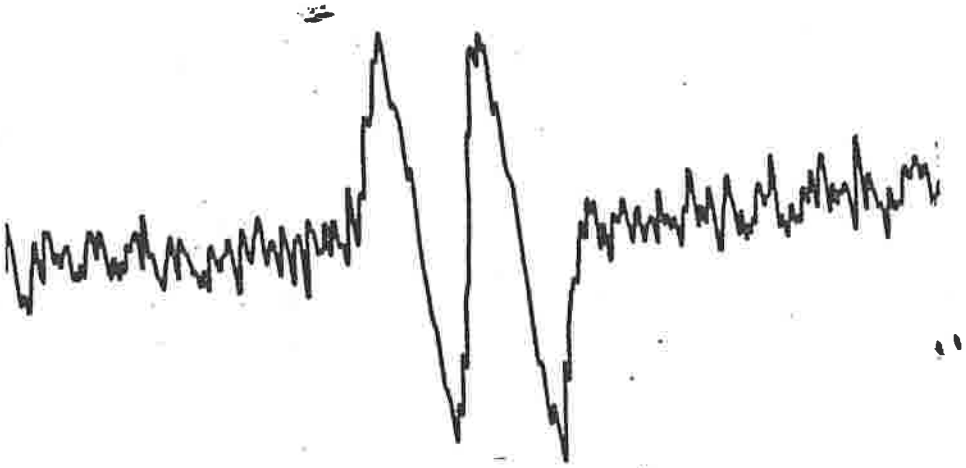
|--------->

0.45 MT

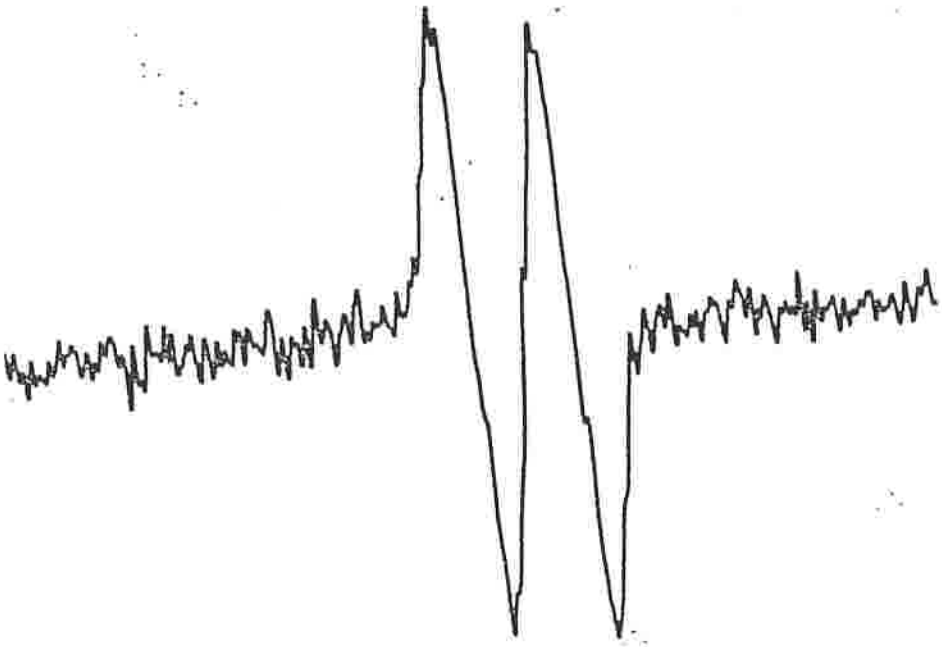
Hyperfine splitting constants are: a_N; 1.37mT a_H; 0.16 mT.

FIGURE 6.2: TYPICAL PRE AND POST-EXERCISE ESR SPECTRA OF ASCORBYL RADICAL IN PLASMA SUPPLEMENTED WITH ASCORBIC ACID.

PRE-EXERCISE



POST-EXERCISE



|----->

0.129 MT

Hyperfine splitting constant is a_H: 0.17 mT.

TABLE 6.2: THE EFFECT OF VITAMIN C SUPPLEMENTATION ON BLOOD ASCORBIC ACID CONCENTRATION.

Pre-supplementation ($\mu mol.L^{-1}$)	Post-supplementation ($\mu mol.L^{-1}$)
31.28 ± 7.77	117.54 ± 8.96 **p= 0.005**

NB: The p value is for Wilcoxon matched pairs test. The level of significance was set at the $p< 0.05$, level.

Table 6.2 clearly shows the effect of oral ascorbic acid supplementation on the plasma ascorbic acid concentration. Supplementation with 1 gram of ascorbic acid 2 hours before the exercise test resulted, as expected, in a statistically significant increase in plasma ascorbic acid concentration.

FIGURE 6.3: THE EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON PLASMA ASCORBIC ACID CONCENTRATION.

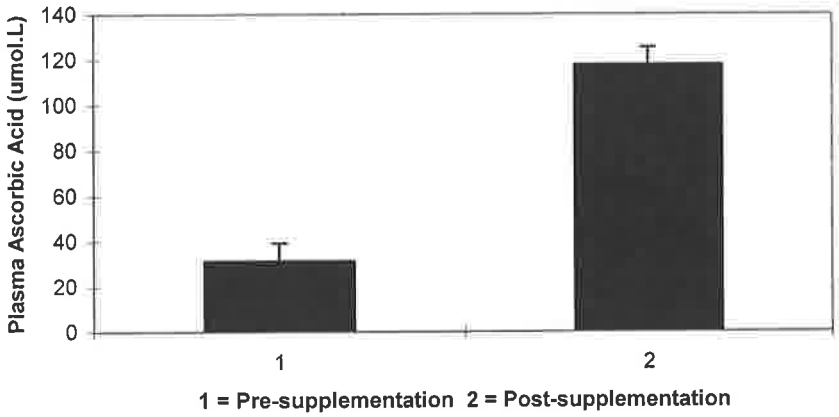


Figure 6.2 shows the effect of supplementation with 1 gram of ascorbic acid on plasma ascorbic acid concentration. Supplementation resulted in a statistically significant increase in blood ascorbic acid concentration p=0.005.

TABLE 6.3: THE EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON LIPID PEROXIDATION AND BLOOD INDICES OF OXIDATIVE DAMAGE FOLLOWING EXHAUSTIVE AEROBIC EXERCISE.

	PRE-EXERCISE	POST-EXERCISE
PBN adducts (Arbitrary units)	0.02 ± 0.01	0.04 ± 0.02 **ns**
Ascorbyl radical (Arbitrary units)	0.01 ± 0.06	0.01 ± 0.01 **ns**
MDA ($\mu mol.L^{-1}$)	0.63 ± 0.07	0.68 ± 0.05 **ns**
LH ($\mu mol.L^{-1}$)	1.12 ± 0.21	1.12 ± 0.08 **ns**
Endotoxin ($Eu.ml^{-1}$)	<0.01 ± 0.00	<0.01 ± 0.00 **ns**

NB: Results are expressed as mean, ± SEM. Both parametric and non-parametric tests revealed no significant difference post vs. pre-exercise. MDA, malondialdehyde; LH, lipid hydroperoxides; ns, not significant.

Table 6.3 shows that acute ascorbic acid supplementation resulted in a noticeable attenuation of free radical increase in the plasma of healthy human subjects following exhaustive aerobic exercise as shown by a lack of significant increase post-exercise. Furthermore this is coupled with a complete abolition of endotoxin from the venous circulation. The implication is therefore that exercise-induced systemic endotoxaemia is free radical mediated, and that exercise-induced free radical increase may be prevented by prior supplementation with vitamin C.

TABLE 6.4: THE EFFECT OF VITAMIN C SUPPLEMENTATION ON BLOOD ANTIOXIDANT STATUS.

	PRE-SUPPLEMENTATION	POST-SUPPLEMENTATION
TAC ($\mu mol.L^{-1}$ Trolox eq)	510.5 ± 45.1	1680 ± 36.1 **p= 0.005**
Ascorbic acid ($\mu mol.L^{-1}$)	31.28 ± 7.77	117.54 ± 8.96 **p= 0.005**
α-tocopherol ($\mu mol.L^{-1}$)	15.42 ± 3.76	16.01 ± 2.21 **ns**
β-carotene ($\mu mol.L^{-1}$)	0.10 ± 0.02	0.08 ± 0.01 **ns**
α-carotene ($\mu mol.L^{-1}$)	0.01 ± 0.04	0.03 ± 0.01 **ns**
Retinol ($\mu mol.L^{-1}$)	1.45 ± 0.41	1.46 ± 0.10 **ns**
Lycopene ($\mu mol.L^{-1}$)	0.30 ± 0.12	0.57 ± 0.09 **ns**

NB: Results are expressed as mean, ± SEM. TAC, total antioxidant capacity Trolox equivalents.

Table 6.4 demonstrates that supplementation with ascorbic acid causes a statistically significant increase in plasma ascorbic acid concentration. While the lipid-soluble antioxidants, such as α-tocopherol, unsurprisingly remain unchanged. Plasma TAC also increased significantly following ascorbic acid supplementation which is indicative of the contribution this vitamin makes to the total plasma antioxidant potential.

TABLE 6.5: THE EFFECT OF VITAMIN C SUPPLEMENTATION ON BLOOD ANTIOXIDANT STATUS FOLLOWING EXHAUSTIVE AEROBIC EXERCISE.

	PRE-EXERCISE	POST-EXERCISE
TAC ($\mu mol.L^{-1}$) Trolox eq	1680 ± 36.1	1710 ± 59.1 **p=0.004**
Ascorbic acid ($\mu mol.L^{-1}$)	117.54 ± 8.96	121.90 ± 10.00 **p=0.005**
α-tocopherol ($\mu mol.L^{-1}$)	16.01 ± 2.21	17.59 ± 2.40 **p=0.005**
β-carotene ($\mu mol.L^{-1}$)	0.08 ± 0.01	0.09 ± 0.02 **ns**
α-carotene ($\mu mol.L^{-1}$)	0.03 ± 0.01	0.03 ± 0.01 **ns**
Retinol ($\mu mol.L^{-1}$)	1.46 ± 0.10	1.69 ± 0.11 **ns**
Lycopene ($\mu mol.L^{-1}$)	0.57 ± 0.09	0.64 ± 0.09 **ns**

NB: Results are expressed as mean, ± SEM. TAC, total antioxidant capacity Trolox equivalents. The p-value is for Wilcoxons signed rank test for paired data.

Table 6.5 shows that maximal aerobic exercise has in general no effect on the lipid-soluble antioxidants in healthy subjects supplemented with 1 gram of ascorbic acid, although α-tocopherol did increase significantly post-exercise. An explanation for this apparently spurious result may be that exercise causes efflux of antioxidants from tissues into the bloodstream or that ascorbic acid regenerated vitamin E in response to an oxidative challenge. Plasma TAC and ascorbic acid concentration did increase significantly following maximal aerobic exercise. This may be expected since it has been shown that antioxidant supplementation results in siginificant increases post-exercise (Maxwell et al 1993). Thus these results are in agreement with published reports and may reflect efflux of antioxidants from tissues in response to oxidative stress in this case induced by maximal exercise.

TABLE 6.6: COMPARISON OF RESULTS OF STUDY 1 (AEROBIC EXERCISE) AND STUDY 3 (AEROBIC EXERCISE AND VITAMIN C SUPPLEMENTATION).

	STUDY 1		STUDY 3	
	Pre-exercise	**Post-exercise**	**Pre-exercise**	**Post-exercise**
PBN adducts	0.04 ± 0.01	0.18 ± 0.04 **p = 0.003**	0.02 ± 0.01	0.04 ± 0.02 **ns**
Ascorbyl radical	0.02 ± 0.001	0.03 ± 0.002 **p = 0.04**	0.01 ± 0.06	0.01 ± 0.01 **ns**
MDA ($\mu mol.L^{-1}$)	0.70 ± 0.05	0.80 ± 0.04 **p = 0.0125**	0.63 ± 0.07	0.68 ± 0.05 **ns**
LH ($\mu mol.L^{-1}$)	1.15 ± 0.07	1.63 ± 0.29 **p = 0.006**	1.12 ± 0.21	1.12 ± 0.08 **ns**
Endotoxin ($Eu.ml^{-1}$)	0.16 ± 0.03	0.24 ± 0.06 **p = 0.001**	<0.01 ± 0.00	<0.01± 0.00 **ns**
TAC($\mu mol.L^{-1}$ Trolox equiv)	518 ± 38.4	574 ± 51.9 **ns**	1680 ± 36.1	1710 ± 59.1 **p=0.004**
Ascorbic acid ($\mu mol.L^{-1}$)	18.44 ± 5.25	21.71 ± 5.12 **ns**	117.54 ± 8.96	121.90 ± 10.00 **p=0.005**
VO_2 uptake ($ml.kg.min^{-1}$)	6.73 ± 1.08	49.40 ± 2.73 **p< 0.05**	7.98± 1.21	47.43 ± 1.95 **p< 0.05**

NB: Results are expressed as mean, ± SEM. The p values are for Wilcoxon matched pairs test or Students t-test. * ESR arbitrary units.

Table 6.6 (on the previous page) shows the results of studies 1 and 3 where the same group of subjects performed an identical maximal aerobic exercise (VO_{2max}) test. These data demonstrate an attenuation of the parameters of oxidative

damage and also present novel results regarding the attenuation of the post-exercise ESR signal of the PBN adduct in healthy human plasma supplemented with ascorbic acid. Also a complete abolition of plasma endotoxin concentration is observed. The mechanisms of the effect of ascorbic acid are discussed further in section 6.4, but probably involve scavenging of free radicals by antioxidant ascorbic acid.

INTER ASSAY CORRELATIONS.

In the present study there was no correlation between the ESR parameters (PBN adduct; ascorbyl radical) and maximal oxygen uptake. There was no correlation between lipid peroxidation and ESR, or between lipid peroxidation and maximal oxygen uptake. There was no significant inverse correlation between antioxidant status and oxidative damage. This may be due to small sample numbers and/or large standard deviations.

6.4 DISCUSSION.

The purpose of the present study was to examine the effect of an antioxidant intervention on free radical production and indices of oxidative damage following exhaustive aerobic exercise eliciting maximum oxygen uptake.

The administration of an acute dose of 1 gram L-ascorbic acid resulted in an attenuation of exercise-induced oxidative damage. There were no significant increases in any of the ESR parameters. Vitamin C prevented the exercise-induced increase in lipid peroxidation previously reported in study 1. Vitamin C also completely abolished endotoxin from the plasma pre and post-exercise.

THE EFFECT OF ORAL ASCORBIC ACID SUPPLEMENTATION ON PRE AND POST-EXERCISE ESR SPECTRA.

There was no significant post-exercise increase in any of the ESR parameters in the present study, which suggests that ascorbic acid is an effective antioxidant in the prevention of exercise-induced oxidative stress.

The hyperfine coupling constants recored from the ESR spectra of the PBN adducts were a_N = 1.37 mT and a_H = 0.16 mT. These values compare favourably to the coupling constants (a_N = 1.35 to 1.37 mT, a_H = 0.20 to 0.225 mT) reported by Mergner et al (1991) who suggested that the radicals were oxygen-derived alkoxyl radicals. This is supported by the hyperfine values ranging from: a_N = 1.36 mT and a_H = 0.19 mT to; a_N = 1.41 mT and a_H = 0.42 mT, found in blood reported by Tortolani et al (1993) who also suggest the species are alkoxyl or carbon-centred radicals. While Garlick et al (1987) reported almost identical values of a_N = 1.36 mT and a_H = 0.156 mT in reperfused rat heart and attributed the species as either carbon-centred or alkoxyl radicals formed via reaction of primary oxygen-centred radicals with membrane lipids. This is supported by data from Ashton et al (1998) who reported the detection of oxygen-centred radicals (a_N = 1.371 mT and a_H = 0.194 mT) in the blood of subjects following exhaustive aerobic exercise. They also suggested that the species were probably secondary alkoxyl radicals derived intitially from primary oxygen-centred radicals following peroxidation of membrane lipids. Dickens et al (1991) reported the detection of alkoxyl radicals (a_N = 1.375 mT and a_H = 0.225 mT), the authors ascribed the origin of the adducts as being derived from membrane phospholipids. Thus it is fair to say that the radical species detected in the present study, while present in smaller quantities, are probably the same species as the PBN adducts detected in study 1.

Ascorbic acid supplementation resulted in a 50% decrease in the baseline or resting ESR signal of the PBN adduct when compared to study one. Thus it can be said that ascorbic acid attenuated the resting level of free radical production by 50% as measured by ESR. There was however a non-significant increase in the post-exercise ESR signal following vitamin C supplementation suggesting that ascorbic acid did not completely inhibit free radical production induced by maximal aerobic exercise. This lack of complete inhibition of exercise-induced oxidative damage is in agreement with Kanter et al (1993) who studied MDA levels pre and post-exercise. Thus ascorbic acid prevented the statistically significant increase in the concentration of the PBN adduct post-exercise reported in study 1.

Importantly however the levels achieved post-exercise in the vitamin C intervention study were the same as the resting ESR signal in the un-supplemented study. Thus the dramatic increase in post-exercise ESR signal intensity is not seen following ascorbic acid supplementation. Therefore although 1 gram of ascorbic acid does not entirely inhibit exercise-induced free radical production it did prevent the three to four fold increase of ESR signal observed in study 1. The mechanism of action of the antioxidant properties of ascorbic acid involves direct interaction and scavenging of aqueous lipid-derived peroxyl radicals and chain breaking in lipid peroxidation (Bendich et al 1986), combined with indirect antioxidant properties by regenerating vitamin E from the tocopheroxyl radical (Niki et al 1983, Niki 1987), while ascorbic acid is itself regenerated by glutathione (Meister 1992). The two-step reversible oxidation of ascorbic acid yields dehydroascorbic acid with ascorbyl radical as an intermediate.

The delocalised nature of the unpaired electron on the ascorbyl radical makes it comparatively unreactive but it can react with other free radicals thereby

preventing propagation of radicals and lipid peroxidation, and terminating the process. The identification of the species as probably being secondary alkoxyl radicals derived from lipid peroxidation of cell membranes by primary oxygen-centred radicals and their location in the aqueous phase of the blood provides an explanation of the effectiveness for vitamin C in the present study.

The hyperfine coupling constants are similar to those reported in study 1, thus indicating that the species are the same, ie. of biological origin. However, a possible limitation of the use of coupling constants is that there is an element of overlap in the values obtained which prevents accurate identification of the species trapped and is thus a limitation of ESR in this setting.

The ascorbate free radical was again, as expected, detected in the samples in the present study with a hyperfine coupling constant of a_H = 0.17 mT which is in agreement with study 1. This is supported by Sharma and Buettner (1993) who reported an identical ESR signal (a_H = 0.18 mT) in human blood plasma subjected to continuous oxidative stress. The concentration of the ascorbyl radical did not change significantly following exhaustive aerobic exercise and vitamin C supplementation which indicates attenuation of exercise-induced free radical production. This is at variance with the results of study 1, where a significant post-exercise increase in the ascorbyl radical was noted in the plasma of unsupplemented subjects. An explanation for this is that increases in the plasma ascorbyl radical signal is a sensitive indicator of increased oxidative stress *in vivo* (Buettner and Jurkiewicz 1993). Increased ascorbyl radical signal reflects consumption of ascorbic acid as it is oxidized while acting in an antioxidant capacity, thus indicating increased levels of oxidative stress and response by the host antioxidant defence mechanism (Tomasi et al 1989). ESR evidence reported by Kihara et al (1995) indicates that the ascorbyl radical is produced in significantly increased amounts during oxidative stress, and that antioxidant

treatment with superoxide dismutase and catalase attenuated the increase in ascorbyl radical concentration. This therefore implicates superoxide as a possible source of the increased signal amplitude of the PBN adduct and ascorbyl radical observed in study 1. It can therefore be said that the lack of change in post-exercise ascorbyl radical signal intensity is due to the high plasma level of ascorbic acid thus providing ample defence against exercise-induced free radical production.

The most common aqueous radical is the hydroperoxyl ($HOO^{\cdot}$) radical which is generated in equilibrium with the superoxide anion and is scavenged by ascorbic acid (Wayner et al 1987). Ascorbic acid may make a relatively greater contribution to the plasma antioxidant defence mechanism than vitamin E (Wayner et al 1987). In a randomised placebo controlled trial, supplementation with 1 gram of ascorbic acid was shown to enhance the plasma total radical-trapping antioxidant potential of young healthy University students (Mulholland and Strain 1993). Thus since the species detected by ESR are probably aqueous lipid-derived radicals eg. alkoxyl radicals, then increased plasma levels of ascorbic acid would scavenge any radicals produced by exercise. Furthermore increases in plasma antioxidant concentration have been reported post-exercise in supplemented subjects (Maxwell et al 1993), thus the present study is in agreement with literature reports since it demonstrates statistically significant increased levels of ascorbic acid and total antioxidant capacity post-exercise.

In the present study the exercise protocol was identical to that of study 1 and time to exhaustion and VO_{2max} were similar between study 1 and this study. Respiratory exchange ratio was identical in both studies at termination of the test indicating an equal level of intensity and similar metabolism in both studies.

A twelve percent reduction in oxygen consumption and improvements in mechanical efficiency in vitamin C supplemented subjects compared to controls during exercise has been reported, with saturation of vitamin C body pools significantly increasing efficiency in athletes (Howald et al 1975). Furthermore enhanced aerobic working capacity and time to exhaustion occurred in vitamin C supplemented subjects performing cycle ergometer exercise (Howald et al 1975). This indicates a greater capacity for work at lower absolute oxygen levels, and increased capacity at a relative percentage of VO_{2max} possibly as a result of improved cardiovascular efficiency.

Similarly vitamin C has been shown to increase endurance capacity in guinea pigs while tissue consumption of vitamin C was also increased (Gerster 1989). This suggests that as exercise progresses and oxygen flux continues vitamin C is consumed by the tissues, presumably acting as an antioxidant and scavenging the excess free radicals produced during exercise.

THE EFFECT OF ORAL ASCORBIC ACID SUPPLEMENTATION ON EXERCISE-INDUCED LIPID PEROXIDATION.

Vitamin C supplementation attenuated the previously noted increase in lipid peroxidation post-exercise. This is in agreement with other workers who have reported an attenuation of lipid peroxidation following strenuous exercise (see section 2.3.3). In humans and rodents antioxidant vitamins have been shown to reduce levels of lipid peroxidation, prolong endurance, and prevent oxidation of blood glutathione following a variety of exercise tests (Kanter et al 1993, Gohil et al 1986, Novelli et al 1990, Sastre et al 1992).

In the present study the resting level of plasma MDA was 10% lower than in the unsupplemented study. While the post-exercise level was 15% lower compared

to the unsupplemented subjects. This demonstrates the effectiveness of ascorbic acid in attenuating resting as well as exercise-induced lipid peroxidation. The mechanism by which ascorbic acid is effective at inhibiting lipid peroxidation and may involve regeneration of vitamin E and possible scavenging of secondary radicals that may cause lipid peroxidation.

Supplementation with 1 gram of vitamin C was shown to inhibit production of thiobarbituric acid reactive substances (TBARS) production in the lipoproteins of healthy male and female subjects (Rifici and Khachadurian 1993). While dietary vitamin C decreases endogenous protein oxidative damage, malondialdehyde (MDA) and maintains fatty acid unsaturation in the guinea pig liver (Barja et al 1994). This is evidence of the effect of ascorbic acid at the aqueous-lipid interface presumably where it is regenerating vitamin E and scavenging secondary aqueous lipid-derived radicals.

Alessio et al (1997) have recently demonstrated that exercise-induced oxidative stress was highest in a group of nine (n=9), healthy, physically active male subjects when they did not supplement with vitamin C. Furthermore acute supplementation with 1 gram of ascorbic acid inhibited lipid peroxidation compared to placebo and chronic ascorbic acid supplementation. The data of Alessio et al (1997) support the effectiveness of ascorbic acid supplementation as shown in the present study.

Frei et al (1988) have suggested that a simple controlled regimen of ascorbic acid supplementation may prove helpful in preventing formation of lipid hydroperoxides (LH) which might not be detoxified by endogenous plasma activities thus causing damage to critical targets. The baseline level of LH was 2% lower in the present study compared to the unsupplemented study. Whereas the post-exercise concentration was 31% lower in this study. Thus indicating that 1

gram of vitamin C inhibits exercise-induced lipid peroxidation, and supresses baseline production of MDA and LH. Furthermore ascorbic acid has been described as an outstanding antioxidant in human blood plasma (Frei et al 1989).

THE EFFECT OF ORAL ASCORBIC ACID SUPPLEMENTATION ON PLASMA ANTIOXIDANT STATUS FOLLOWING STRENUOUS AEROBIC EXERCISE.

There was a statistically significant post-exercise increase in plasma ascorbic acid and TAC concentration. However as expected, the pre-exercise ascorbic acid concentration in study 1 was less than that observed following supplementation. Furthermore, the post-exercise plasma concentration in study 1 was only 17% of the post-exercise level in the present study. This would enable the plasma to mount an effective defence against the increased amounts of free radicals produced during the maximal oxygen uptake exercise test.

Robertson et al (1991) reported an increase in blood antioxidant status in response to training load. While physical exercise may lead to release of ascorbic acid from the adrenal gland and concomitant shift in the distribution of vitamin C, leading to an increased metabolic turnover of this vitamin (Fishbaine and Butterfield 1984). This is supported by Gleeson et al (1987) who suggest that exercise induces a change in the distribution, metabolism and excretion of ascorbic acid. Thus the post-exercise increase in ascorbic acid concentration and plasma TAC is in agreement with the literature and may reflect an antioxidant response to an oxidative challenge.

Reznick et al (1992) have stated that administration of vitamin C may reduce oxidative damage caused by exercise, which has been demonstrated in this study. The increased ascorbic acid concentration of the blood plasma in the present study appears to have served as an effective antioxidant in preventing

the exercise-induced increase in oxidative damage previously reported in the literature and in study 1.

However, α-tocopherol also increased significantly post-exercise in this study ($p=0.005$). It is difficult to offer an explanation for this apparently spurious result but one interpretation is that the increase may reflect efflux from tissues in response to stress, in this case exercise. It may also be due to the increased levels of ascorbic acid regenerating vitamin E allowing accumulation in the plasma. All other antioxidant parameters including: α-carotene; β-carotene; lycopene remained unchanged by exercise in ascorbic acid supplemented subjects.

THE EFFECT OF ORAL ASCORBIC ACID SUPPLEMENTATION ON PLASMA LIPO-POLYSACCHARIDE CONCENTRATION FOLLOWING STRENUOUS AEROBIC EXERCISE.

The concentration of plasma endotoxin (lipo-polysaccharide) did not increase following maximal aerobic exercise. Furthermore the administration of 1 gram vitamin C completely abolished endotoxin production to below the detection limit, both before and after exercise. This implicates oxidative damage to the gut mucosa as the source of increased endotoxin observed in the unsupplemented study. During exercise blood is diverted from the gastro-intestinal tract to the working muscles in order to provide the required oxygen and fuel substrates to perform work. The gut is thus in a state of relative ischaemia. On cessation of exercise blood flow is restored and the gut is reperfused, this can result in the generation of several ROS including: hydrogen peroxide; superoxide anion; and hydroxyl radical.

These oxidants cause damage to the gut mucosa allowing trans-location of bacterial endotoxin into the systemic circulation. This may be one possible reason for the increase in fever and infection observed following exercise of a strenuous

nature (Hemila 1996, Heath et al 1991, Witt et al 1992). Also increased levels of superoxide which may occur during reperfusion, have been shown to result in the accumulation of lipid peroxidation by-products which jeopardize cell membrane integrity and lead to cell death (Alessio and Goldfarb 1988).

In addition to this, strenuous exercise has been shown to have a negative effect on immune function (Nieman 1994), which depresses cytokines interleukin-1 and interleukin-2 thus affecting reactions involving natural killer cells (Shephard et al 1995). Therefore exercise may be synonymous with stress (Weiss 1989).

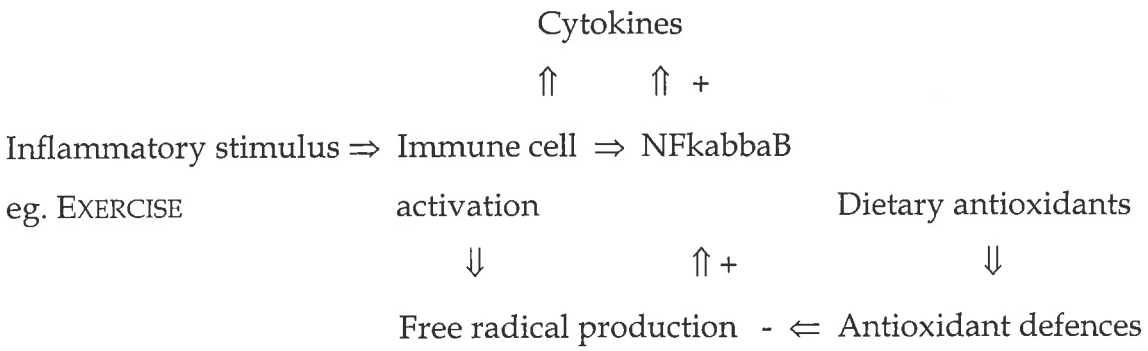
Endotoxin has been shown to increase the numbers of macrophages, neutrophils and lymphocytes in bronchoalveolar lavage fluid (Nagai et al 1995), while exercise alters the acute phase immune response (Conn et al 1995). Endotoxaemia however triggers release of cytokines that can cause hypotension, fever, shock (endotoxic shock) and even death; recently endotoxin levels have been shown to be elevated in athletes requiring medical attention after strenuous cycling (Moore et al 1995). Therefore while strenuous exercise may cause a decrease in the cytokine levels, if this exercise causes endotoxin to be translocated into the bloodstream cytokines may be released causing inflammation, fever, hypotension, and possibly endotoxic shock.

Vitamin C supplementation has been shown to reduce the incidence of post-race symptoms of upper respiratory tract infections in ultramarathon runners (Peters et al 1993). The strong interdependence between micronutrient status and immunocompetence has led to the suggestion that appropriate antioxidant intervention may lead to a decrease in the risk of infection (Schmidt 1991).

The ability to maintain adequate antioxidant defence during the cytokine mediated response to infection (or perhaps endotoxin release), is important in

preventing the response from disadvantaging the host (Grimble 1995). The mechanism of this protection is outlined below (see figure 6.2).

FIGURE 6.4: MECHANISM OF ANTIOXIDANT PROTECTION FROM FREE RADICAL MEDIATED PRO-INFLAMMATORY CYTOKINE PRODUCTION.



Adapted from: Grimble (1995).

It can be seen from the above scheme that antioxidant intervention can serve to modulate the immune response to a stressor, in this case exercise, via quenching of free radicals. It is therefore proposed that it is this mechanism that is responsible for the abolition of bacterial lipopolysaccharide from the circulation of subjects supplemented with ascorbic acid reported in the present study.

Vitamin C has been shown to protect cell membranes from auto-oxidation, extracellular ascorbate has been shown to quench superoxide generated by activated neutrophils while the concentration of ascorbate in leukocytes is 200 times that of the erythrocyte (Bendich 1989). This indicates a strong role for antioxidant vitamin C in immunocompetence.

6.5. CONCLUSION.

Increased oxygen flux through intermediate metabolism during exercise increases the rate of oxygen radical production and alters cellular antioxidant status (Packer and Viguie 1989). Exercise participation can itself modulate interactions between nutritional status and immune function, especially via increased intake of antioxidants to protect the active person against an augmented production of free radicals due to increased tissue metabolism and minor muscle injuries (Shephard and Shek 1995). While it has been suggested that prior vitamin C supplementation may exert a protective effect against eccentric exercise-induced muscle damage in humans (Jakeman and Maxwell 1993).

The present study demonstrates a decrease in all parameters associated with oxidative damage and an enhancement of the antioxidant defences in healthy human subjects performing maximal aerobic exercise. It demonstrates the inhibition by ascorbic acid supplementation of the ESR signal and free radical-mediated lipid peroxidation products in human blood post-exercise. It also demonstrates the abolition of endotoxin from blood plasma by ascorbic acid supplementation during maximal aerobic cycle ergometer exercise.

These results may have implications for current advice regarding diet and physical activity though due to sample numbers, they should be viewed with caution when attempting to extrapolate to a wider audience. Additionally it is worth repeating this study in subjects for whom physical exercise is recommended in the management of a pathology, but where the pathology itself is implicated in increased levels of free radicals eg. cystic fibrosis. It can be concluded therefore that prior supplementation with ascorbic acid is effective in diminishing exercise-induced oxidative stress.

Chapter Seven

IN VITRO STUDIES

7.0 INTRODUCTION.

The aim of this study was to attempt the confirmation of the identity of the radical species detected in the previous studies and to draw some conclusions regarding their origin. The mechanism of the pathway of origin *in vivo* may well involve some or all of the following series of reactions shown overleaf (figure 7.0). Figure 7.0 shows that as exercise increases whole body oxygen uptake/flux increases. Thus it can be assumed that the flow of electrons down the mitochondrial electron transport chain also increases with amplification of leakage and concomitant ROS production. Mitochondrial ROS production by exercise may well be associated with the 100-200 fold increase in oxygen flux in individual skeletal muscle fibres (Strain 1995), leading to the formation of superoxide. Superoxide may react with nitric oxide, released in increased quantities from the vascular endothelium during exercise, leading to the formation of the damaging peroxynitrite. Alternatively, the possibility exists that superoxide is dismuted to yield hydrogen peroxide, leading to the formation of the hydroxyl radical via Fenton chemistry. This then abstracts a hydrogen atom from cell membrane polyunsaturated fatty acid (PUFA) such as arachidonic acid leading to the formation of a secondary oxygen-derived alkoxyl radical and/or carbon-centred radical. Carbon-centred radicals can undergo molecular rearrangement to form a conjugated diene with the concomitant production of aqueous peroxyl radicals. Ascorbic acid can scavenge these peroxyl radicals leading to attenuation of the products of lipid peroxidation (as shown in study 3). Alternatively the pathway may procede leading to the formation of lipid hydroperoxides and concomitant alkoxyl radical production with termination by ascorbic acid. Lipid peroxidation may be inititated by any species that has sufficient reactivity to abstract a hydrogen atom from a memrane PUFA side chain such as arachidonic and linolenic acid (Aruoma 1994), as shown in figure 7.0.

FIGURE 7.0: SUGGESTED POSSIBLE PATHWAY OF SECONDARY RADICAL FORMATION.

EXERCISE

⇓

INCREASED O_2 UPTAKE/FLUX

⇓

INCREASED FLOW OF ELECTRONS DOWN ELECTRON TRANSPORT CHAIN

⇓

INCREASED LEAKAGE OF ELECTRONS FROM CHAIN/PARTIAL REDUCTION OF O_2

FORMATION OF PRIMARY OXYGEN CENTRED RADICAL EG. $O_2^{\cdot -}$

⇓ ⇓

HYDROGEN ABSTRACTION BY $^{\cdot}OH$ FROM $O_2^{\cdot -}$ PRODUCTION

CH_2 GROUP OF CELL MEMBRANE PUFA ⇓ ⇓ $\Leftarrow H_2O_2$

⇓⇐**ASCORBIC ACID SCAVENGING** PEROXIDATION FENTON

FORMING CARBON CENTRED RADICAL (-$^{\cdot}$CH-) ⇓ REACTION

OR ALKOXYL R$\cdot$ ⇓ $O_2^{\cdot -} + NO^{\cdot}$ ⇓ $^{\cdot}OH$

MOLECULAR REARRANGEMENT FORMING ⇓

CONJUGATED DIENE $ONOO^{\cdot -}$

⇓ $\Leftarrow O_2$

PEROXYL RADICAL

⇓

PROPAGATION OF LIPID PEROXIDATION AND FORMATION OF LH

⇓

ALKOXYL RADICAL FORMATION

CYCLIC PEROXIDE AND ENDOPEROXIDE FORMATION

⇓

FORMATION OF AQUEOUS HYDROPEROXYL RADICALS AND MDA

⇓

FORMATION OF LIPID-DERIVED ALKOXYL RADICALS ⇐ **ASCORBIC ACID TERMINATION**

Peroxidation of membrane PUFA yields lipid-derived radicals and products of lipid peroxidation such as lipid hydroperoxides and malondialdehyde. Decomposition of lipid hydroperoxides may also yield alkoxyl radicals (Tortolani et al 1993). PUFA by virtue of their double bonds are particularly susceptible to hydrogen abstraction and concomitant oxidant damage. Those with greater numbers of double bonds such as arachidonic acid (C20:4, n-6) are therefore especially vulnerable.

The peroxidation of membrane PUFA by biologically significant oxygen-centred radicals during oxidative damage is central to the *in vitro* studies and has been confirmed by several authors. Bielski et al (1983) has stated that the conjugate acid of $O_2^{\cdot-}$, the perhydroxyl radical ($HOO^{\cdot}$) reacts with linoleic acid at a biologically significant rate that occurs in the absence of transition metal ions, which may be a cause of superoxide toxicity *in vivo*. Furthermore the perhydroxyl radical initiates fatty acid peroxidation by two parallel lipid hydroperoxide (LH) pathways; LH dependent and LH independent (Aikens and Dix 1991).

It is feasible that the LH-dependent pathway may be relevant to lipid peroxidation initiation *in vivo*. The spin trapping of carbon-centred radicals from linoleic acid and arachidonic acid has been demonstrated *in vitro* (Iwahashi et al 1991). Davies (1989) using the spin trapping technique, has demonstrated peroxyl, alkoxyl carbon-centred adduct formation independent of reducing equivalents in rat liver microsomal fractions. He further speculates that haem-dependent free radical production observed in the study may be a significant factor in the cytotoxicity of lipid hydroperoxides (Davies 1989).

The mechanism of radical formation and antioxidant quenching was elucidated by Zhu et al (1990). The authors identified the addition of molecular oxygen to

carbon-centred, lipid derived radicals forming peroxyl radicals. The radicals were quenched by several antioxidants including vitamin E, however the rate of stable antioxidant radical formation was lower for unsaturated fatty acids when compared to saturated lipids. While it is also known that ascorbic acid is able to scavenge aqueous lipid-derived peroxyl radicals (Bendich et al 1986). In order to attempt to identify and define the origin of the ESR signal of the PBN adduct in this thesis *in vitro* studies are warranted.

7.1 METHODS.

EXPERIMENT ONE.

These experiments were performed in order to attempt to identify the PBN adduct observed during the *in vivo* studies and also to attempt to draw conclusions regarding the origin of the said ESR signals, since it is hypothesised that the signals observed during the *in vivo* studies are secondary alkoxyl radical species.

1. Arachidonic acid (97%) pure in semi-aqueous soluble form was purchased from Sigma Ltd. UK.
2. Nine (9 mg) milligrams of arachidonic acid was dissolved in 3 ml of de-ionised water [3mg.ml].
3. This lipid-aqueous mixture was allowed to auto-oxidize in a warm water bath, at 37 $^{\circ}$C for 2 hours.
4. A 140 mmol.L^{-1} solution of the spin trap α-phenylbutyl-tert-nitrone (PBN) was used in this experiment using the same protocol as in the main studies.

5. At 2 hours, 1.5 ml of this PBN solution was added to the arachidonic acid solution and allowed to auto-oxidize for a further 2 hours.
6. On completion of the incubation period 3 ml of the solution containing PBN/arachidonic acid was removed from the glass vial into a separate vial.
7. To this solution 3ml HPLC grade toluene was added and vortex mixed for 30 s.
8. ESR analysis was then performed using the procedure described in section chapter 3.

NB: This experiment was repeated in a darkened room such that the arachidonic acid solution was not exposed to light. In addition to this identical control experiments were also performed where ESR analysis was carried out on samples that had no PBN added.

EXPERIMENT TWO.

These experiments were performed in order to determine the effects of a water soluble antioxidant (ascorbic acid), on a lipid-aqueous model system. The objective being to draw conclusions regarding the effectiveness of the antioxidant properties of ascorbic acid in a lipid-aqueous system that may represent conditions similiar to those found *in vivo*. It is hoped that these data will help explain why ascorbic acid, a water-soluble antioxidant, inhibited lipid peroxidation *in vivo* as reported in study 3.

1. Samples were prepared as above, experiment 1.
2. Ascorbic acid, pure white crystalline form (Sigma Ltd.), was dissolved in 3 ml de-ionised H_2O to a concentration of 100 $\mu mol.L^{-1}$. This concentration

approximates the plasma levels achieved in the supplemented human studies.

3. After 2 hours of auto-oxidation, to 3 ml of the arachidonic acid-aqueous solution, 1.5 ml of the 100 $\mu mol.L^{-1}$ ascorbic acid solution was added.
4. This final solution was allowed to stand in a warm water bath at 37° C for a further 2 hours.
5. ESR analysis was carried out using identical procedures and conditions from experiment one.
6. Blank experiments were also performed.

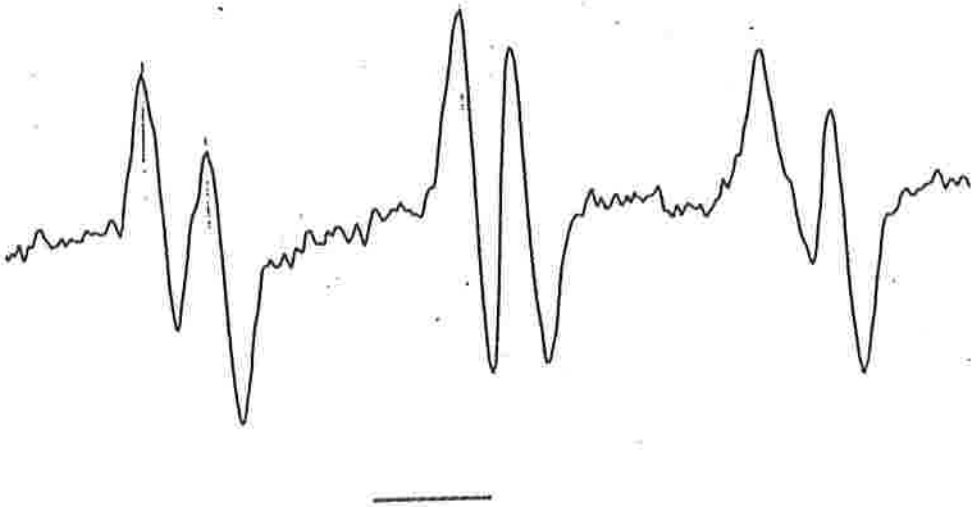
ESR CONDITIONS.

All samples were analysed on the same JEOL RE2-X series X-band spectrometer. The spectrometer is equipped with a TE 110 cavity, and 100 KHz frequency modulation. Room temperature ESR analysis was performed using the following operating conditions: Frequency, 9.431 GHz; Power, 10 mW; Field centre, 334.6 mT or 333.6 mT for aqueous flat cell; Scan width, ± 4.0 mT; Sweep time, 4.0 min; Modulation amplitude, 0.1000 mT; Gain, 2500; Time constant, 0.30 s; Accumulations, 5. During analysis of the de-gassed, toluene extracted samples precision bore quartz ESR tubes were used. During analysis of the ascorbyl radical an aqueous flat cell was used. All glassware was prepared specifically for these studies and were not used for any other experiments.

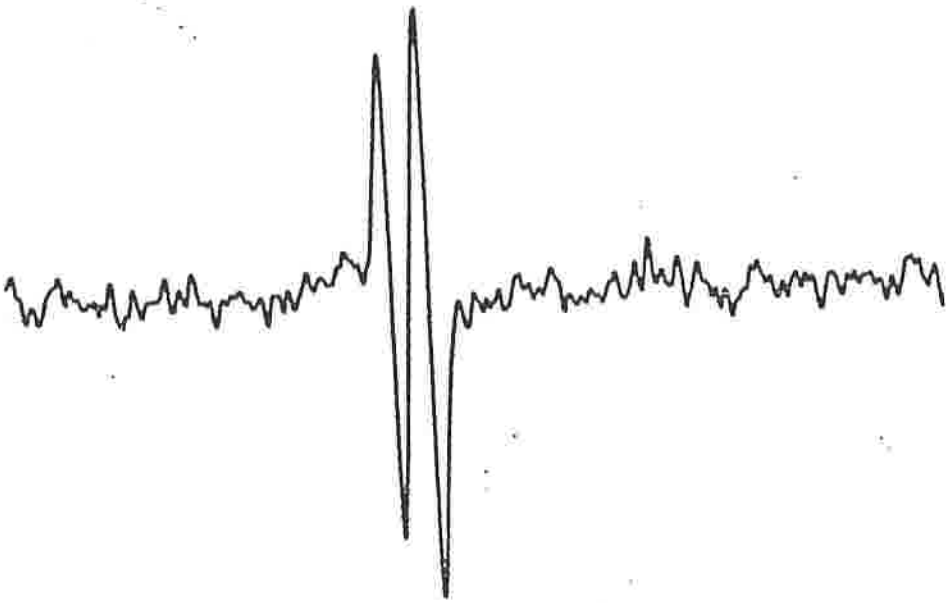
7.2 RESULTS.

The ESR spectra overleaf show the formation of a triplet of doublets characteristic of the nitroxide adduct of PBN. Importantly the signal was completely abolished by the addition of ascorbic acid approximating to physiological concentrations.

FIGURE 7.1: ESR SPECTRA OF PBN ADDUCT AND ASCORBYL RADICAL IN ARACHIDONIC ACID SOLUTION BEFORE AND AFTER ADDITION OF ASCORBIC ACID.



0.45 mT



0.129 mT

Hyperfine splitting constants for the PBN adducts were: a_N, 1.50 mT a_H, 0.27 mT. The splitting constant for the ascorbyl radical was: a_H, 0.175 mT.

7.3 DISCUSSION.

The ESR spectra show a triplet of doublets characteristic of a nitroxide spin adduct, which is similar to those observed during the *in vivo* studies. The hyperfine splitting constants are consistent with the trapping by PBN of secondary oxygen and carbon-centred radicals such as alkoxy and alkyl radicals formed by the reaction of oxygen with polyunsaturated lipids (Bolli et al 1988). The hyperfine coupling constants seen in the present study (a_N = 1.50 mT, a_H = 0.27 mT) are in agreement with those reported by Iwahashi et al (1991) for arachidonic acid-derived radicals (a_N = 1.58 mT, a_H = 0.22 mT). However, the nitrogen splitting is slightly higher than those reported in the *in vivo* studies, as is the hydrogen splitting. (Hyperfine coupling constants from the *in vivo* studies are: study one, a_N = 1.371, a_H = 0.194 mT; study two, a_N = 1.350 mT, a_H = 0.190 mT; and study three, a_N = 1.37 mT, a_H = 0.16 mT). One explanation for this is that the ESR spectra of the PBN adduct appears to show some evidence of line broadening which could influence the values obtained and may be due to the presence of oxygen in the system.

The addition of ascorbic acid into the system completely abolished the ESR signal of the arachidonic acid-derived PBN adduct and replaced it with the ascorbyl radical (a_H = 0.17 mT). This is consistent with the antioxidant properties of ascorbic acid. Vitamin C is known to be a potent chain-breaking antioxidant which can inhibit free radical initiated lipid peroxidation (Liu et al 1996). Similar findings have been reported by several other groups.

Niki et al (1984) demonstrated the ability of ascorbic acid to inhibit oxidation of methyl linoleate via trapping of peroxyl radicals and reduction of the tocopheroxyl radical back to vitamin E. Furthermore vitamin C was preferentially consumed before vitamin E, and changes in vitamin E were noted

only after vitamin C was exhausted. This was confirmed in later studies by Sharma and Buettner (1993). They reported preferential oxidation of ascorbic acid over vitamin E which was demonstrated using ESR by the appearance of increased concentrations of the ascorbyl radical (a_H = 0.18 mT) in response to oxidative stress. Laroff et al (1972) reported ESR evidence of the oxidation of ascorbic acid by the hydroxyl radical. Furthermore the scavenging effect of vitamin E is maintained only if ascorbic acid is present (Scarpa et al 1984).

Doba et al (1985) demonstrated the effective inhibition of lipid peroxidation initiated in the aqueous phase of multi-lamellar phospholipid liposomes, they state that each ascorbate molecule terminates 0.6 radical chains. This is supported by evidence from Niki et al (1985) who demonstrated that vitamin C is an effective antioxidant, scavenging aqueous phase radicals and being preferentially oxidized before vitamin E.

The ESR spectra illustrate the presence of aqueous lipid-derived radicals from arachidonic acid, a known constituent of cell membranes and a potent producer of free radicals *in vivo* by a lipoxygenase system. These data support the identification of the radical species trapped in the human studies as being secondary radicals probably derived from the peroxidation of membrane PUFA by primary oxygen centred radicals. A criticism however, of the *in vitro* studies is the use of arachidonic acid as a model of *in vivo* lipid peroxidation in this setting. If this study were to be repeated a phospho-lipid system would be used which is an accepted model of biological membranes (Scarpa et al 1984). Although this may not yield information to determine which lipid is specifically providing an ESR signal. Furthermore it would be prudent in future studies to determine the ESR spectra of polyunsaturated fatty acids with different numbers of double bonds including; linoleic and linolenic acid containing two and three double bonds respectively. These may field different ESR coupling constants enabling

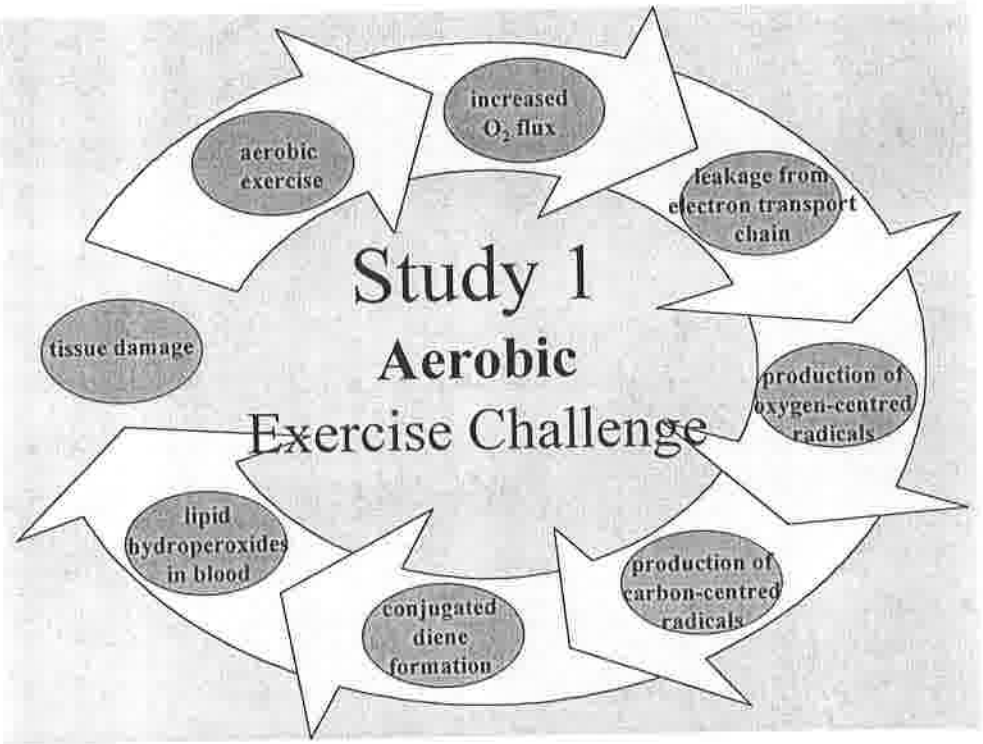
inferences to be made regarding the origin of the signals *in vivo*. Additionally stimulation of lipid peroxidation by enzymatic oxidation may also further serve to increase the specificity of the spectra obtained since auto-oxidation can result in non-specific and different rates of peroxidation of lipids in membrane systems.

In summary the free radical species trapped by PBN in the human exercise studies are likely to be derived from cell membrane PUFA following increased oxidant production by exercise and may be cautiously identified as oxygen-derived alkoxyl radicals. However, the possibility that the species are carbon-centred radicals derived from β-scission of alkoxyl radicals cannot be excluded.

Chapter Eight

GENERAL DISCUSSION AND OVERALL CONCLUSIONS

FIGURE 8.0: SIMPLIFIED MECHANISM OF INVOLVEMENT OF OXYGEN IN THE GENERATION OF RADICAL SPECIES FOLLOWING EXHAUSTIVE EXERCISE.



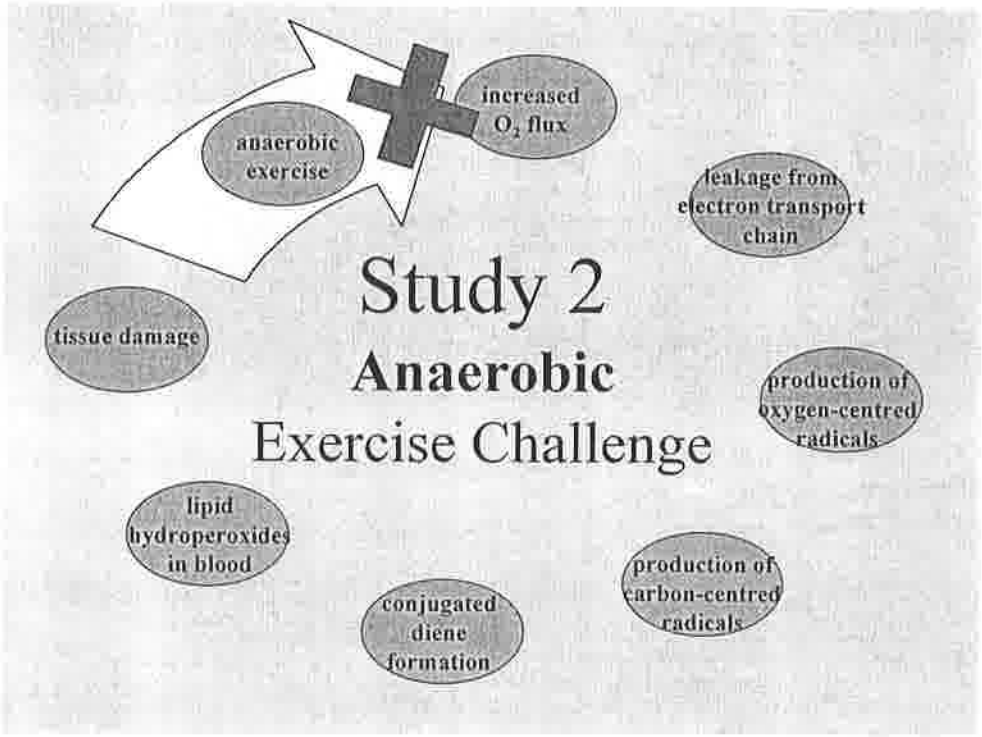
Study 1 demonstrated a statistically significant increase in free radicals following exhaustive aerobic exercise as evidenced by increases in the concentration of the PBN adduct and ascorbyl radical in the venous circulation of healthy humans. Using the hyperfine coupling constants of the ESR spectra, it is cautiously suggested that the identity of the radical species trapped by PBN in study 1 are secondary alkoxyl radicals radicals derived from peroxidation of membrane PUFA by primary oxygen-centred radicals. However, the possibility that the species are carbon-centred radicals which may be formed via β-scission of alkoxyl radicals cannot be excluded in such a complex biochemical system.

In addition to this, the concentration of the ascorbyl radical increased significantly in the plasma following exhaustive aerobic exercise (study 1). This is potentially important since the ascorbyl free radical has been suggested to be a sensitive marker of oxidative stress *in vivo*. This again supports the hypothesis that strenuous aerobic exercise leads to oxidative stress.

This conclusion is supported by increases in products of free radical-mediated lipid peroxidation (lipid hydroperoxides and malondialdehyde) observed in the blood of the subjects in study 1. In study 1 the plasma concentration of both measurements of free radical-mediated lipid peroxidation increased significantly following maximal aerobic exercise. This is further supported by a lack of significant increase in blood antioxidant status post-exercise in study 1. Although it should be stated that there appeared to be a trend to increased blood antioxidant status following maximal aerobic exercise, which suggests an oxidative challenge and an antioxidant response to this challenge via efflux of antioxidants from the tissues into the blood.

The conclusion that increased oxygen flux is implicated in the observed increases in ESR parameters and lipid peroxidation in study 1, is supported by results from study 2 examining the production of free radicals in human serum following intense anaerobic exercise.

FIGURE 8.1: SIMPLIFIED MODEL OF THE LACK OF DEPENDENCE ON OXYGEN FLUX TO GENERATE FREE RADICALS DURING ANAEROBIC EXERCISE.



Study 2 involved anaerobic exercise and hence does not lead to increased whole body oxygen flux and concomitant enhancement of electron leakage. Figure 8.1 shows a simplified model of the lack of dependence on oxygen flux to generate free radicals during intense anaerobic exercise. The results from study 2 showed that intense anaerobic exercise does not result in increased concentration of free radicals in blood as shown by a lack of significant increase in the PBN adduct. Additionally the performance of intense anaerobic exercise did not result in any significant increase in the plasma concentration of the ascorbyl radical. During intense anaerobic exercise energy for muscular work comes from 'anaerobic' sources such as the creatine phosphate and glycolytic pathways, with little increase in electron flux down the electron transport chain. Since leakage of electrons from the mitochondrial

electron transport chain is known to occur then it is feasible that the large increases in whole body oxygen uptake observed in study 1 is responsible for the increased ESR signal post-exercise seen in study 1. Furthermore the fact that mean heart rate increased to only approximately 60% of the age-predicted maximum heart rate in study 2 supports the 'oxygen flux' hypothesis, since it was reported in study 1 that a threshold of increase in free radical concentration exists. This threshold may correspond to approximately 74% of maximal oxygen uptake.

This is further supported by the lack of increase in the plasma concentration of major products of free radical-induced lipid peroxidation, namely LH, following the Wingate anaerobic exercise test in study 2. However plasma MDA did increase significantly in study 2. One possible explanation of this confounding result is that inadequacies in clearance of MDA led to accumulation in the plasma. However a possible criticism of study 2 is that there was an increase in the mean heart rate during and immediately after the Wingate anaerobic test suggesting an aerobic contribution to this test.

Increased mean heart rate following the Wingate anaerobic exercise test may be one explanation for the trend to slight but, non-significant, increases in the levels of the PBN adduct in the blood following the Wingate test. These data thus support the hypothesis that aerobic exercise leads to increased free radical production in the venous circulation of healthy humans, as measured by ESR spectroscopy.

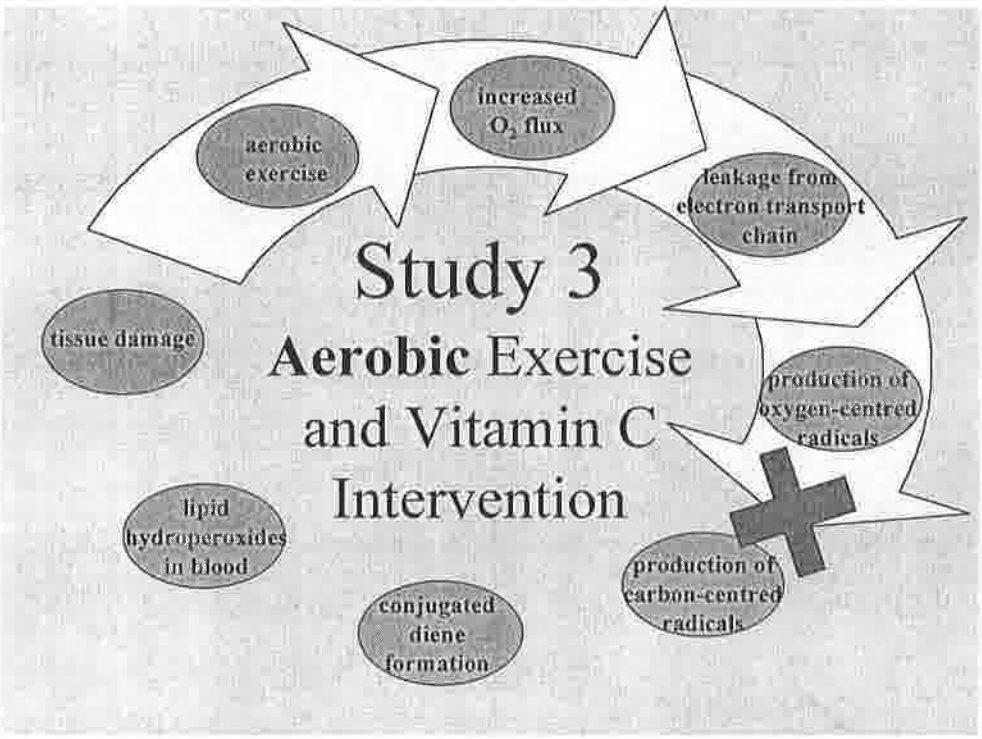
In addition to demonstrating the importance of oxygen flux in the generation of increase amounts of free radicals, results from study 1 demonstrated, as previously mentioned, the possible existence of a 'threshold' of exercise-induced oxidative stress corresponding to approximately 74% maximal oxygen uptake. The work also provided information on the relative intensity of exercise at which this threshold is exceeded, thus leading to oxidative

stress. This is potentially clinically relevant since a lack of physical activity is implicated in the aetiology and progression of several pathologies. Thus if a 'free radical threshold' during exercise is identified then exercise prescription below this threshold would allow improved cardiovascular benefits without the potentially deleterious effects associated with increased free radical production as demonstrated by increased plasma endotoxin concentration and free radical-mediated lipid peroxidation reported in this thesis.

Study 3 showed that ascorbic acid supplementation attenuated the post-exercise increase in the ESR signal intensity (amplitude) of the venous circulation. The levels of the PBN adduct and the ascorbyl radical detected in blood were decreased by administration of vitamin C. The results of study 3 support the hypothesis that strenuous aerobic exercise leads to increased free radical production, thus vitamin C is an effective antioxidant in preventing exercise-induced free radical production.

Study 3 also demonstrated that supplementation with ascorbic acid, as expected, significantly increases the plasma ascorbic acid concentration. This may be important since ascorbic acid status of males is low compared to females. Supplementation with ascorbic acid also results in an attenuation of the post-exercise increase in lipid peroxidation products of oxidative damage such as MDA and LH following maximal aerobic exercise. Figure 8.2 overleaf presents a simplified model of the possible mechanism by which ascorbic acid prevents increases in oxidative damage following aerobic exercise. Ascorbic acid is a potent water-soluble antioxidant which would be expected to quench free radicals formed as a result of aerobic exercise as shown in figure 8.0. Therefore ascorbic acid intervention in subjects undertaking an aerobic exercise challenge should attenuate any increases in free radical production as shown by a lack of increase in ESR parameters and indirect supporting assays including LH, MDA, and endotoxin.

FIGURE 8.2: POSSIBLE MECHANISM BY WHICH ASCORBIC ACID PREVENTS OXIDATIVE DAMAGE FOLLOWING AEROBIC EXERCISE.



This research also demonstrated the abolition of bacterial endotoxin by supplementation with vitamin C from the plasma of healthy subjects following maximal short term aerobic exercise. This is potentially clinically important since exercise is known to cause an acute phase immune response resulting in fever and release of cytokines. Raised levels of cytokines have been implicated in infection and collapse following extreme endurance exercise. Furthermore raised levels of endotoxin have been observed in our laboratory in the blood of patients with meningococcal meningitis (Dr. Simon Jackson - unpublished observation). Additionally administration of endotoxin to rodents has been shown to cause oxidative stress as measured by ESR via increased levels of the ascorbyl radical (Stark et al 1988). The data in the

thesis support the hypothesis that exercise-induced systemic endotoxaemia is a free radical-mediated phenomenon.

The work in this thesis has applied the most sensitive, specific and direct method to measure this phenomenon, ie. ESR spectroscopy, which enables the investigation of transient free radical species. The use of ESR is not however without difficulty, in particular the inability to categorically identify the radical species trapped by PBN. It is suggested therefore that if the work were to be repeated an attempt should be made to use electron nuclear double resonance (ENDOR) spectroscopy in addition to ESR to attempt to categorically identify the radical species. A limitation of this however is that the sensitivity is decreased 10-fold (Dr. Chris Rowlands - personal communication). Thus with the low concentrations of free radicals seen in blood post-exercise this may not be possible.

Jackson (1996) has suggested that whilst a considerable amount of work has been performed in this area basic data are still lacking to answer the following questions:

- Are free radicals produced in excess during exercise ?
- Is any excess free radical production during exercise damaging or beneficial to tissues ?
- Does antioxidant supplementation reduce free radical activity during exercise ?
- Does antioxidant supplementation during exercise have beneficial effects on tissues ?

It is suggested that the evidence contained in this thesis contributes, at least in part, to answering these important questions. The evidence would suggest that free radicals are produced in excess during aerobic exercise as a result of

increases oxygen flux and leakage of electrons from the electron transport chain. Furthermore that these free radicals mediate tissue damage. However, antioxidant supplementation attenuates the ESR signal intensity, prevents increases in the indirect supporting indices of oxidative damage and is thus beneficial to the tissues.

Specifically the research has shown that exercise leads to increases in free radical concentration in the blood of healthy subjects as shown by direct ESR measurements and indirect supporting assays of free radical-mediated lipid peroxidation. It has also shown that this may be damaging to tissues due to for example, the translocation of lipopolysaccharide into the venous circulation, which may be linked to fever following exercise. Furthermore the research has demonstrated that antioxidant supplementation reduces free radical activity during exercise, which may be beneficial.

A positive aspect of this research is the fact that it has sought to include several indices, both direct and indirect, of free radical activity in a complex biological system. This should allow conclusions to be drawn regarding the acceptability of the evidence contained in this thesis this is not always the case in the published literature.

However, it is difficult to make categoric statements regarding whether or not the increased production of these potentially damaging species is harmful to the tissues, since it may be a physiological response to stress, in this case exercise. Thus this work would suggest a cause-and-effect relationship between exercise and free radical production and by implication an association between free radical production and tissue damage. In view of this association it is considered to be unwise to advocate indiscriminate use of antioxidant supplementation during exercise, although some individuals may require increased antioxidants during exercise.

There are thus certain questions that remain to be answered and certain mechanisms that remain to be elucidated. It is therefore suggested that further research examine the following:

1. The precise dose of ascorbic acid (or tissue concentrations) required to inhibit exercise-induced oxidative stress.

2. The threshold of exercise-induced oxidative stress remains to be elucidated. This is of clinical importance since exercise confers several benefits to health, however exercising above the threshold of free radical production may lead to oxidative damage to critical cellular targets *in vivo*. Thus precise determination of this threshold would permit exercise prescription that enable the positive benefits of exercise to accrue without the deleterious effects of exercise-induced oxidative stress.

3. The precise origin of the ESR signal needs to be determined. This would be of importance since it would determine the nature of exercise required to avoid increased free radical production. It would also yield information as to the critical sites of damage thus allowing a strategy to be developed to allow the positive effects of exercise to predominate.

4. The effect of a moderate aerobic training protocol on the post-exercise ESR signal should be examined since aerobic training is thought to enhance the enzymatic antioxidant defence mechanisms.

5. The free radical response to exhaustive exercise and vitamin C supplementation in a clinical population known to suffer from free radical mediated disorders such as type I diabetes mellitus needs to be determined. Exercise is known to improve blood glucose control and is

therefore recommended in the treatment regime of patients with diabetes mellitus. Thus if vitamin C attenuates exercise-induced oxidative stress in normals then the application to a clinical population is warranted. Furthermore vitamin C is thought to compete with glucose for the same uptake pathway (Levine et al 1996) and may therefore result in improved glucose tolerance.

Thus there is still much work to be performed in this area but it is hoped that this research will provide a useful starting platform. It certainly provides an exercise model and methodology which could be applied to the elucidation of a series of questions around the role of free radicals in exercise-induced oxidative stress, and free radical-mediated disorders such as type 1 diabetes mellitus.

Finally, it may be that strenuous, unaccustomed physical exercise does indeed "profiteth little"!

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APPENDIX 1

BORG SCALE OF RATINGS OF PERCEIVED EXERTION

6

7 Very, very light

8

9 Very light

10

11 Fairly light

12

13 Somewhat hard

14

15 Hard

16

17 Very hard

18

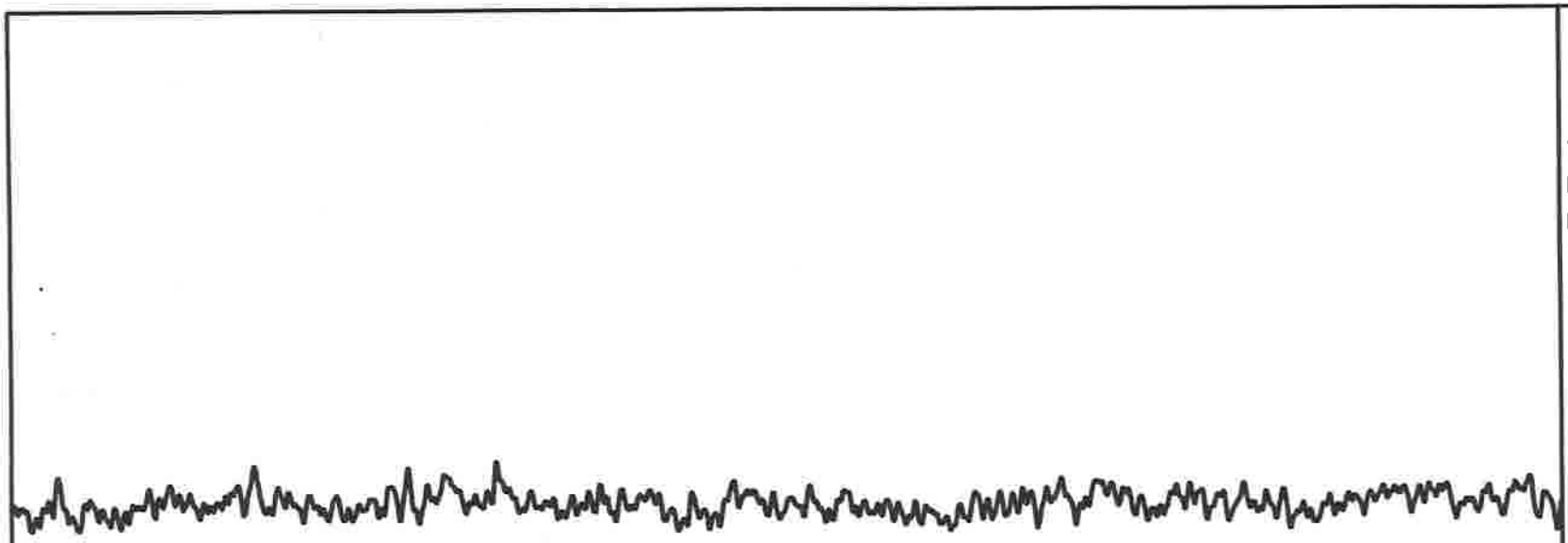
19 Very, very hard

20

APPENDIX 2

ESR SPECTRA OF BLANK (EMPTY) ESR TUBE

05/16/96



ASHTON
NEWTUBE1.EPR

T	300.00	K
Fr	9.4350	GHz
Pw	10.00	mW
Fd	334.7	mT
SW	5.000	mT
ST	2.0	Min
MW	0.10000	mT
G	2500.00	
TC	0.10	sec
Ac	1	

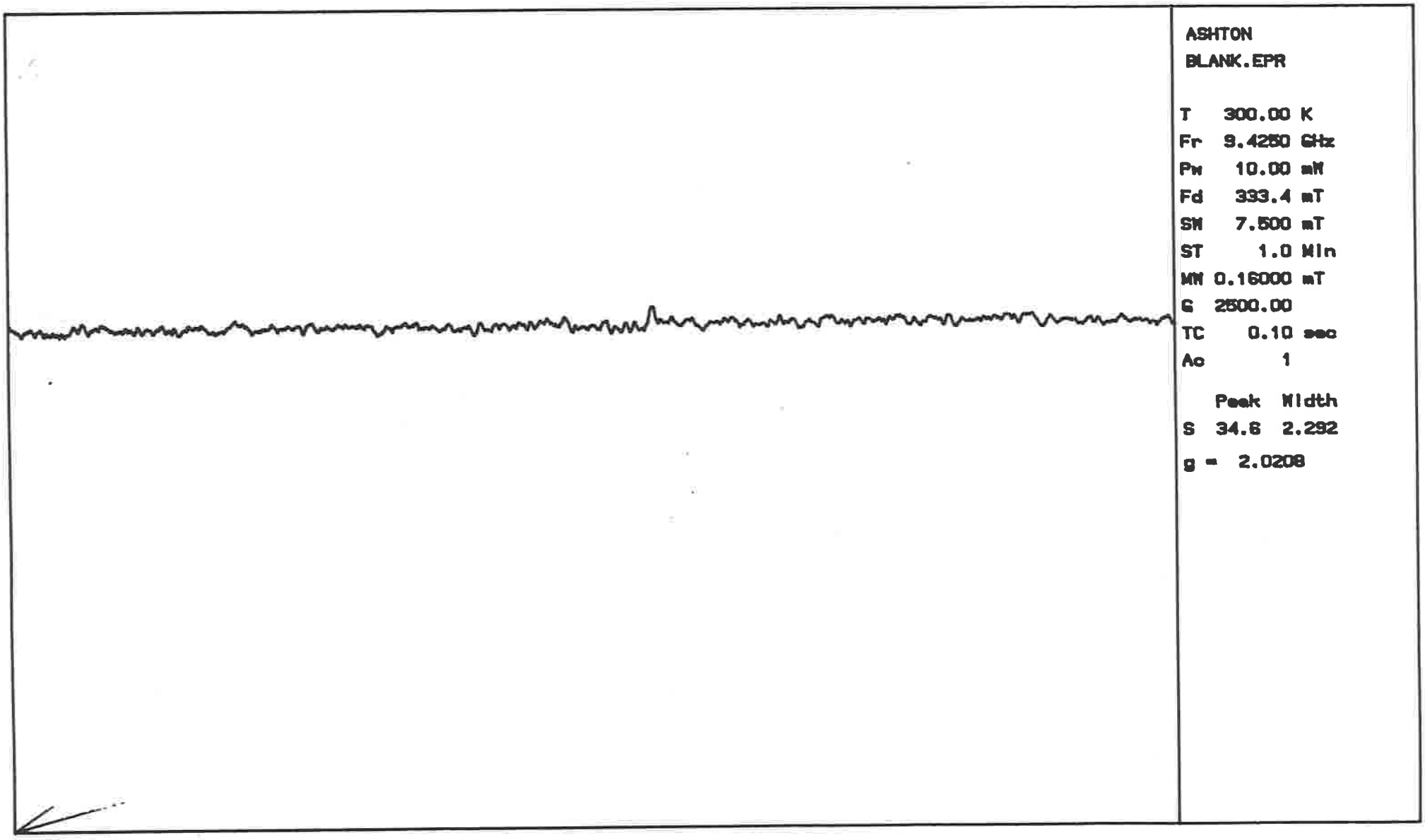
	Peak	Width
S	65.6	2.930

g = 2.0152

APPENDIX 3

ESR SPECTRA OF EMPTY JEOL CAVITY

01/24/96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BLANK.EPR
T 300.00 K
Fr 9.4250 GHz
Pw 10.00 mW
Fd 333.4 mT
SW 7.500 mT
ST 1.0 Min
MW 0.16000 mT
G 2500.00
TC 0.10 sec
Ac 1
Peak Width
S 34.6 2.292
g = 2.0208

APPENDIX 4

ETHICAL APPROVAL FORM

AWDURDOD IECHYD
BRO TAF
HEALTH AUTHORITY

JS/1442

Dr. J.R. Peters,
Consultant Physician,
University Hospital of Wales,
Heath Park,
Cardiff.

Dear Dr. Peters,

96/1442 - The direct measurement of free radicals in biological systems: A pilot study

The Chairman of the Local Research Ethics Committee (Panel A), Mrs K. Fisher has asked me to inform you that your response is satisfactory and your research may now proceed.

Yours sincerely,

C. P

Carl Phillips,
Executive Officer, L.R.E.C.

NHS CYMRU WALES

[illegible]
Churchill House
Churchill Way, Cardiff CF1 4LW
Tel: 01222 229416 Fax: 01222 222307

[illegible]
15 Churchill
[illegible] Churchill, Cardiff CF1 4LW
Tel: 01222 229416 Fax: 01222 222307

Temple of Peace & Health
Cathays Park, Cardiff CF1 3NW
Tel: 01222 231021, Fax: 01222 238606

Lead Healthcare Ltd
Parc Cathays, Cardiff CF1 3NW
Tel: 01222 231021, Fax: 01222 238606

Albert Road
Pontypridd CF37 1LA
Tel: 01443 485122, Fax: 01443 485068

Heol Albert
Pontypridd CF37 1LA
Tel: 01443 485122, Fax: 01443 485068

APPENDIX 5

SUBJECT INFORMED CONSENT FORM

INFORMED CONSENT FORM

Any information contained herein will be treated as confidential. If you answer yes to any of the questions below please give details.

1. Have you had to consult your doctor within the last six months?

* YES / NO

2. Are you presently taking any form of medication?

* YES / NO

3. Do you suffer, or have you ever suffered from:

Asthma	*YES / NO	Diabetes	*YES / NO
Bronchitis	*YES / NO	Epilepsy	*YES / NO

4. Do you suffer, or have you ever suffered from any form of heart disease?

5. Do you currently have any form of injury which may prevent you from completing the tests which have been outlined to you?

* YES / NO

I certify that all procedures have been fully explained by the tester and freely give my consent to take part in these tests.

Signature of Subject: .....

I give my permission for my ward to participate in fitness testing at Cardiff Institute of Higher Education.

Signature of Parent/Guardian/Coach (if U18): .....

Signature of Tester: .....

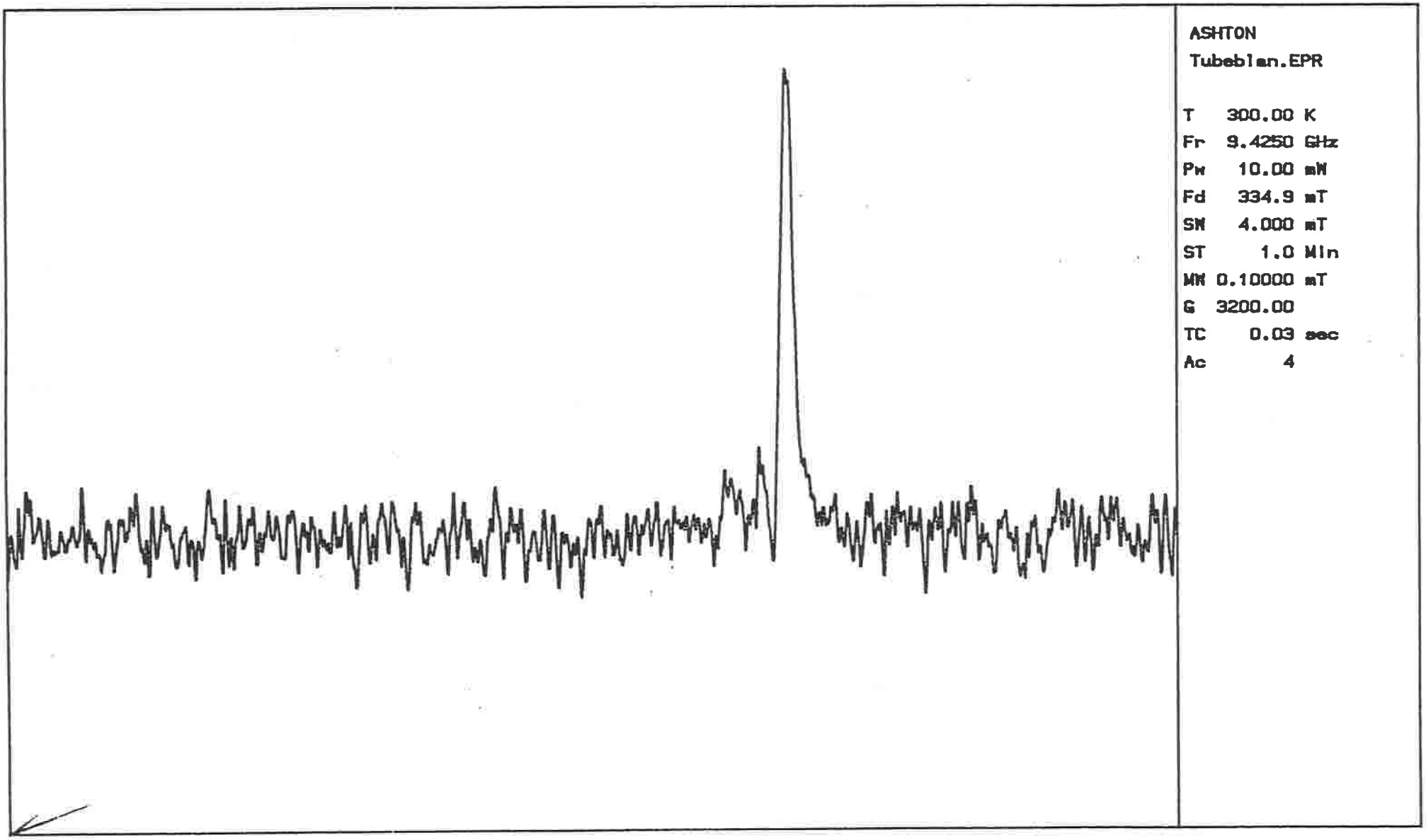
Date:.....

***Please delete as necessary.**

UWIC

APPENDIX 6

ESR SPECTRA OF SCRATCHED TUBE


ASHTON
Tubeblan.EPR
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Fr 9.4250 GHz
Pw 10.00 mW
Fd 334.9 mT
SW 4.000 mT
ST 1.0 Min
MW 0.10000 mT
G 3200.00
TC 0.03 sec
Ac 4

APPENDIX 7

HEALTH QUESTIONNAIRE

QUESTIONNAIRE FOR POTENTIAL PARTICIPANTS IN PROJECTS INVOLVING BLOOD DONATION

Information given will be treated with strictest confidentiality

All questions to be answered by 'ticking' appropriate box

		Yes	No	Not Known
1.	Are you receiving any medicines, dental treatment, have had recent illness or attending hospital outpatients?			
2.	Have you had ears pierced, acupuncture or have been tattooed in the last six months?			
3.	Have you ever been advised by a doctor not to give blood?			
4.	Are you or have you ever suffered from any of the following?			
	Allergy (hay fever, asthma etc)			
	Anaemia or other blood disorders			
	Brucellosis			
	Cancer			
	Diabetes			
	Epilepsy (fits)			
	Glandular fever (in last 2 years)			
	Heart disease			
	Hepatitis (jaundice) or been in contact with a case in last 6 months			
	High blood pressure (except during pregnancy)			
	Kidney disease			
	Peptic ulcers			
	Stroke			
	Thyroid disease (goitre etc)			
	Tropical disease especially malaria			
	Venereal disease			
5.	Is your lifestyle likely to place you at increase risk of HIV infection (AIDS)?			

DECLARATIONS

I have had explained to me, and fully understand, the reasons for donating my blood.

Signed: _____

I have not answered 'yes' to any of the questions listed and to the best of my knowledge am fully eligible to donate blood and do so of my own free will.

Signed: _____ **Dated:** _____

Signature of Phlebotomist: _____ **Dated:** _____

APPENDIX 8

CALIBRATION GRAPH OF MEDRAPHICS ONLINE GAS ANALYSIS SYSTEM

Calibration Report Jun 20, 1996 10:25

Testing Environment

Ambient Temperature	(xC)	:	21.0
Barometric Pressure	(mmHg)	:	755.0
Relative Humidity	(%)	:	0.0
Ambient O2 (computed)	(%)	:	20.93
Ambient CO2 (constant)	(%)	:	0.03

Test Equipment

Syringe Size	(l)	:	3.0
O2 Calibration Gas Concentration	(%)	:	12.0
CO2 Calibration Gas Concentration	(%)	:	5.0
Gas Exchange Valve Dead Space	(ml)	:	80
Minimum Tidal Volume	(ml)	:	150

Gas Exchange Pneumotach

Expiratory			
Calibration Factor	(L/sec/V)	:	1.71
Inspiratory			
Calibration Factor	(L/sec/V)	:	2.18

O2 & CO2 Analyzers

CO2 Phase Delay	(sec)	:	0.37
CO2 Gain Factor	(%/V)	:	1.02
2-90% CO2 Response Time	(sec)	:	0.12
Maximum CO2% During Phase Delay	(%)	:	4.96
O2 Phase Delay	(sec)	:	0.34
O2 Gain Factor	(%/V)	:	9.78
2-90% O2 Response Time	(sec)	:	0.10
Minimum O2% During Phase Delay	(%)	:	12.11

Reviewed by: _____

APPENDIX 9

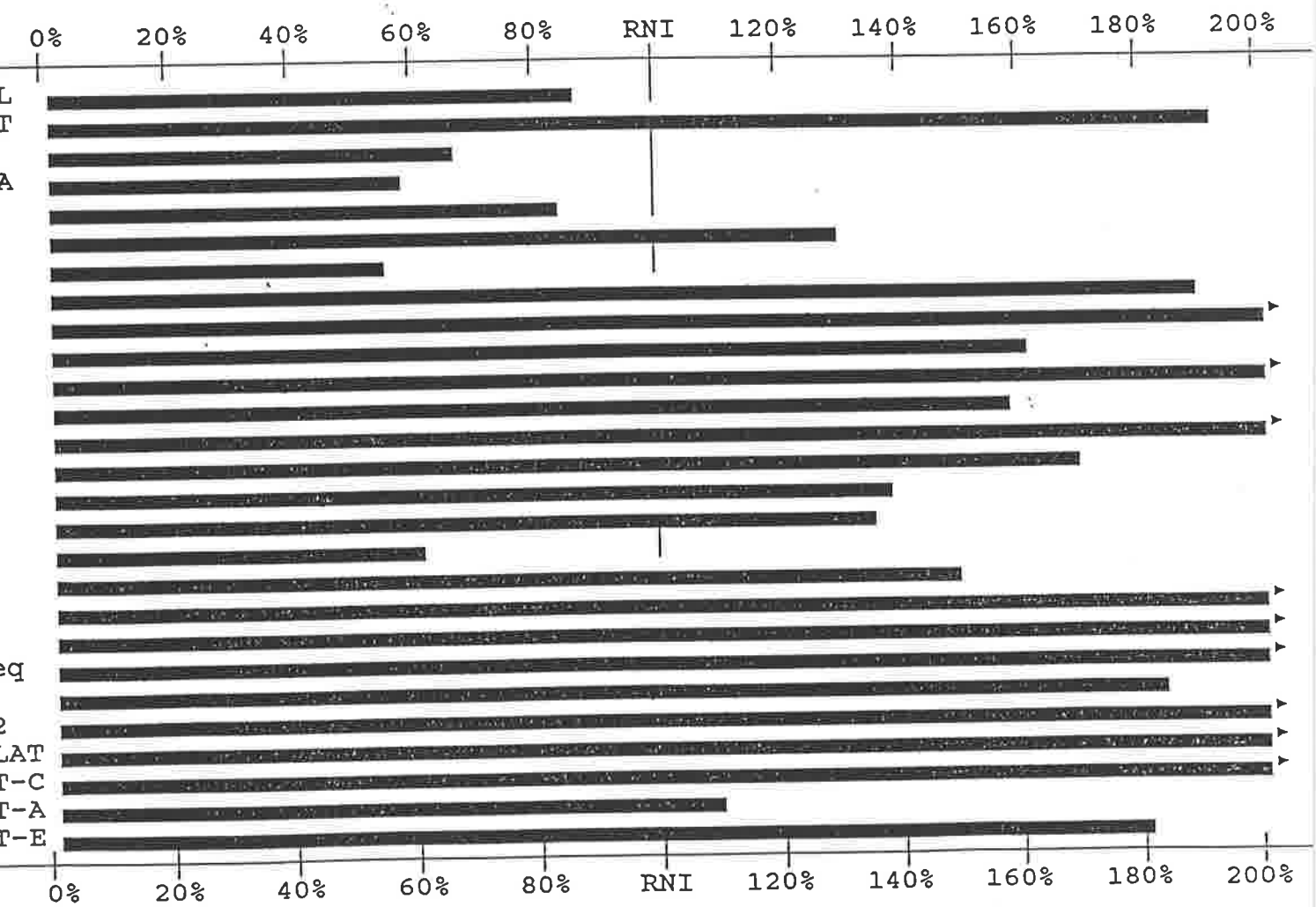
EXAMPLE OF DIETARY HISTORY ANALYSIS

od analysed - Analysis Period: 3 day Profile
ients analysed - Nutrients with UK DRV's
Group - 19-50 years Light-Very Active

al intake figures have been divided by

	-	HEIGHT	- 168.00 cms
	- 23 yrs	WEIGHT	- 69.50 kgms
	- Male		
UPATION	- Light	BMI	- 24.62
ESTYLE	- Very Active		

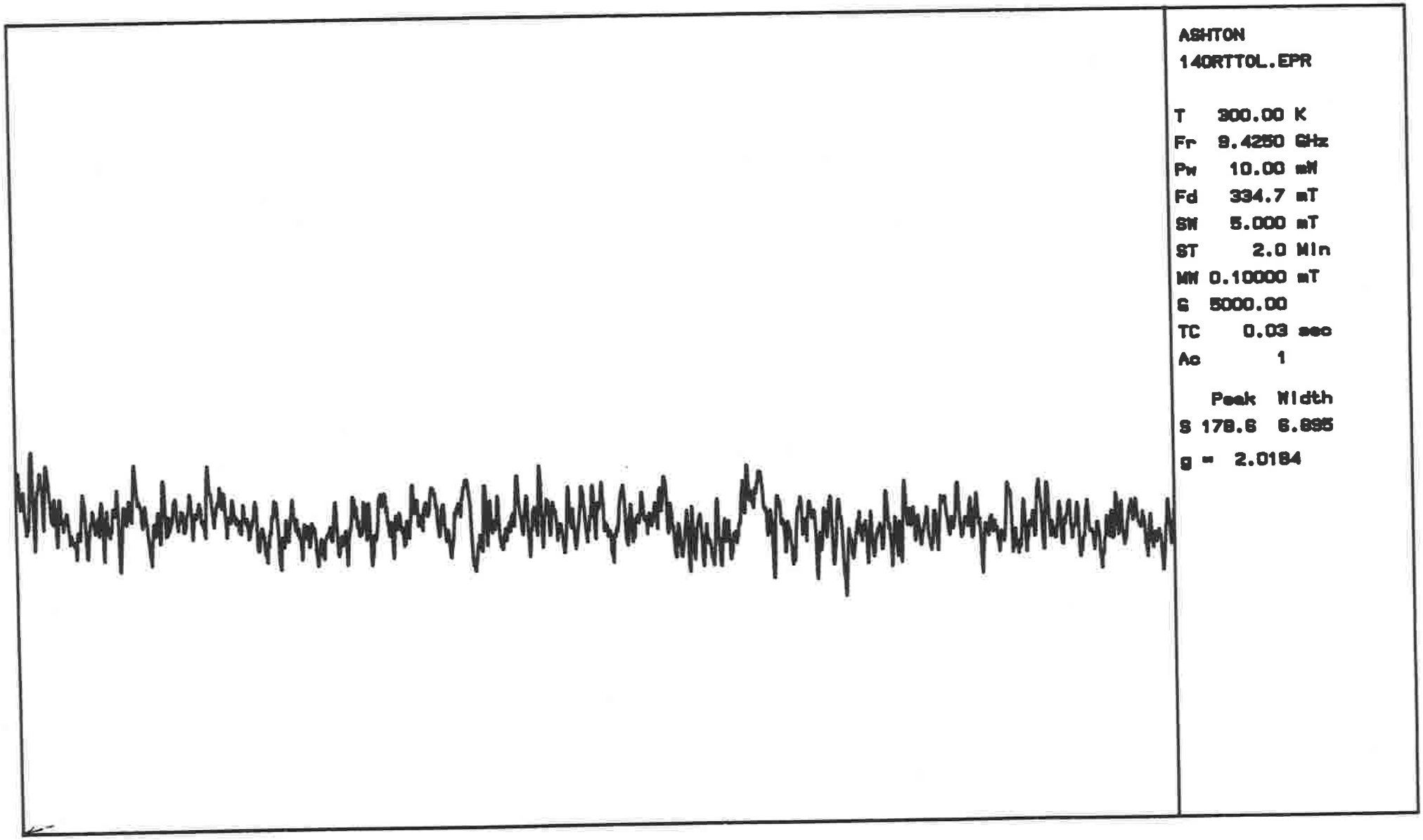
nergy FAT	21.8	% energy CHO	60.1
% energy MUFA	7.8	% energy Starch	24.0
% energy PUFA	3.4	% energy Sugar	36.0
% energy SFA	8.2		
nergy PROTEIN	18.1		
nergy ALCOHOL	0.0		
K ratio	0.7		
as Salt in gms	9.3		



APPENDIX 10

ESR SPECTRA OF TOLUENE

05/09/96



ASHTON
140RTTOL.EPR

T	300.00	K
Fr	9.4250	GHz
Pw	10.00	mW
Fd	334.7	mT
SW	5.000	mT
ST	2.0	Min
MW	0.10000	mT
G	5000.00	
TC	0.03	sec
Ac	1	

	Peak	Width
S	178.6	6.895

g = 2.0184

SCIENTIFIC PUBLICATIONS AND PRESENTATIONS ASSOCIATED WITH THIS WORK

4	2	1	0	7	3	2	B
Journal number			Manuscript Number				

Dispatch: 14.2.98 Journal: EJAPOP
Author Received: No. of pages: 5

Eur J Appl Physiol (1998) ■■: ■■■–■■■

ORIGINAL ARTICLE

Tony Ashton · Christopher C. Rowlands · Eleri Jones
Ian S. Young · Simon K. Jackson
Bruce Davies · John R. Peters

Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise

Accepted: 30 October 1997

Abstract Free radicals or oxidants are continuously produced in the body as a consequence of normal energy metabolism. The concentration of free radicals, together with lipid peroxidation, increases in some tissues as a physiological response to exercise – they have also been implicated in a variety of pathologies. The biochemical measurement of free radicals has relied in the main on the indirect assay of oxidative stress by-products. This study presents the first use of electron spin resonance (ESR) spectroscopy in conjunction with the spin-trapping technique, to measure directly the production of radical species in the venous blood of healthy human volunteers pre- and post-exhaustive aerobic exercise. Evidence is also presented of increased lipid peroxidation and total antioxidant capacity post-exercise.

Key words Electron spin resonance (ESR) spectroscopy · Oxidants · Lipid peroxidation

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Introduction

Oxygen free radicals and other reactive species capable of causing oxidative damage are continuously produced in vivo as a by-product of normal energy metabolism (Alessio 1993). They can produce a variety of damaging effects, including membrane lipid peroxidation, DNA modification, protein oxidation and cell proliferation (Gutteridge and Halliwell 1994). It has been demonstrated recently that people with insulin-dependent diabetes mellitus have increased resting as well as exercise-induced oxidative stress, as indicated by changes in their glutathione redox status (Laaksonen et al. 1996). The biochemical measurement of reactive oxygen species (ROS) has been hampered due to a lack of suitable assays, its low steady-state concentration and the transient nature of ROS and their products. A more routine use of oxidative stress status measurements could be of significant benefit in clinical medicine (Pryor 1993).

It is now accepted that strenuous physical exercise is capable of inducing oxidative stress (Sen et al. 1994), both in an animal (Davies et al. 1982), and a human (Sen 1995) model. Of the many human studies investigating exercise-induced oxidative stress, the majority have relied on indirect indices of free radical damage, such as plasma malondialdehyde (MDA) levels (Lovlin et al. 1987) and serum lipid hydroperoxides (Alessio et al. 1988), demonstrating a post-exercise increase in lipid peroxidation.

Whenever direct evidence of free radical involvement is required, for example in chemical, physiological or biochemical systems, electron spin resonance (ESR) spectroscopy is the method of choice (Asmus and Bonifacic 1994). It is arguably the most sensitive, specific and

T Ashton, E Jones, CC Rowlands, B Davies, IS Young, and JR Peters. The direct measurement of free radicals in human serum following exhaustive exercise: A pilot study. International Society for Free Radical Research, 8th Bi-ennial Conference, Barcelona, Spain. 1996.

T Ashton, E Jones, CC Rowlands, B Davies, IS Young, and JR Peters. Exercise-induced oxidative stress and lipid peroxidation in human serum. 32nd Research Symposium on Oxidants and Antioxidant Therapy in Diabetic Complications. American Diabetes Association. Orlando, Florida. 1996.

T Ashton, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters. The effect of anaerobic exercise on free radical production in human serum. International Electron Spin Resonance (ESR) Spectroscopy Society Annual Conference. Lancaster. 1997.

T Ashton, E Jones, CC Rowlands, B Davies, IS Young, and JR Peters. Electron spin resonance spectroscopic detection of oxygen-centred radicals in human serum following exhaustive exercise. *European Journal of Applied Physiology*. 1998 (In press).

MANUSCRIPTS RECENTLY SUBMITTED AND UNDER PREPARATION:

T Ashton, SK Jackson, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters. Exercise-induced systemic endotoxaemia: The effect of ascorbic acid supplementation. Submitted to *The Lancet*, February 1998.

T Ashton, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters. Exercise-induced free radical production: An ESR investigation of the effect of ascorbic acid supplementation. Under preparation. To be submitted to Free Radical Biology and Medicine.

T Ashton, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters.
The effect of the Wingate anaerobic exercise test on free radical production in human serum. Under preparation. To be submitted to the Journal of Applied Physiology.

FORTHCOMING CONFERENCE PRESENTATIONS:

T Ashton, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters
Exercise-induced systemic endotoxaemia: The effect of ascorbic acid supplementation. International Pathophysiology Society Conference. Kuopio, Finland. June 1998.

T Ashton, CC Rowlands, E Jones, B Davies, IS Young, and JR Peters
Free radical production in young male subjects with type I Diabetes Mellitus: The effect of exercise and ascorbic acid supplementation. Second International Conference on Antioxidants and Anticarcinogens in Nutrition, Health and Disease. Helsinki, Finland. June 1998.