STUDIES TO INVESTIGATE THE ROLE OF FREE RADICALS AND LOW DENSITY LIPOPROTEIN OXIDATION IN THE DEVELOPMENT OF ATHEROSCLEROSIS IN HUMAN SUBJECTS WITH DIABETES MELLITUS.

VOLUME ONE

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SUMMARY

The oxidation of low density lipoprotein (LDL) by free radicals (FR) may be one of the early events in the production of atheroma. I have taken several approaches to test the overall hypothesis that more FR oxidation of LDL occurs in diabetes explaining its associated excess risk of atherosclerosis.

Before embarking on the body of the work a methodological problem was first clarified. Diene conjugated fatty acids (DCFA) are one of the products of FR attack on lipids, and are used as markers of FR activity however dietary fat also contains DCFA. Therefore a study was designed to test the hypothesis that:- diet is an important source of DCFA in human serum and tissues, thereby questioning the use of DCFA as a marker of FR activity. The major DCFA species in human tissues is the 9,11-octadecadieneoic acid isomer [18:2,(9,11)] of linoleic acid [18:2,(9,12)]. Fourteen subjects altered their diets, either increasing ("high diet") or decreasing ("low diet") their intake of foods rich in 18:2,(9,11). Seven day diet histories were kept and scored for their content of 18:2,(9,11). The concentrations of 18:2(9,11) and 18:2(9,12) in serum phospholipids were measured by HPLC with UV detection. The percentage molar ratio (%MR) of 18:2(9,11) to 18:2(9,12) was calculated. It was found that the %MR rose significantly on the "high diet" [1.3(0.4) vs 1.9(0.7); $p=0.01$] and fell significantly on the "low diet" [1.6(0.6) vs 1.1(0.4); $p=0.004$]. Also there was a significant correlation between the change in dietary intake of 18:2(9,11) and the change in the %MR [$r=0.829$; $p=0.001$]. Thus the DCFA content of human tissues is greatly influenced by diet and its use as FR marker limited.

One situation in which diabetes might be associated with more FR oxidation of lipids would be if more FR were generated in diabetes. Since platelet eicosanoid metabolism involves FR intermediates, is altered in diabetes, and platelets have been attributed a role in atherogenesis a study was designed to test the hypothesis that:- platelets are capable of superoxide anion release, this release being greater by the platelets from diabetic subjects. Superoxide release was taken as the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome-C (Fc) measured as the increase in UV absorbence at 550nm. Gel filtered platelet aggregation was induced by shaking platelets with collagen. During 60 minutes exposure to aggregating platelets no Fc reduction attributable to aggregation was seen in the presence or absence of SOD [WITH SOD: 0.013(0.064) vs WITHOUT SOD: 0.012(0.101) OD units, $p=0.987$]. Similar results were obtained at 10 minutes and with resting platelets and no significant differences

existed between diabetic and non-diabetic platelets. Hence platelets do not act as a source of excess FR production in diabetes.

Existence of impaired FR protective mechanisms would be another situation in which more FR oxidation of lipids could occur. Estimating the total peroxyl radical antioxidant parameter (TRAP) a study tested the hypothesis that:- plasma total antioxidant activity, as measured by TRAP, is lower in diabetic subjects than in healthy controls, thereby exposing diabetics to increased risk of FR mediated damage. The TRAP assay measures the inhibition, by plasma, of the peroxidation of aqueous dispersions of linoleic acid, induced by peroxyl radicals generated by thermal decomposition of 2,2' azo-bis [2 amidinopropane hydrochloride]. TRAP was measured in seventeen non-diabetic healthy volunteers and in twenty six diabetic subjects. In the diabetic subjects plasma peroxidisability was also assessed by measuring the increase in the products of lipid peroxidation, namely total diene conjugated (tDC) species and thiobabituric acid reactive species (TBARS), in plasma following incubation for 24 hours at pH 5.5. Thus it was possible to test the further hypothesis that:- the ease with which the lipids from diabetic subjects peroxidise is inversely related to plasma total antioxidant activity, as measured by TRAP. Comparing non-smoking diabetic and non-diabetics no significant difference in TRAP was found (736.5(151.8) vs 754.6(84.3) μmol.l^{-1}, p=0.70) whereas in smokers TRAP was lower in diabetics than non-diabetics (558.5(161.5) vs 838.3(147.5) μmol.l^{-1}, p=0.02) and in diabetics TRAP was lower in smokers than non-smokers (558.5(161.5) vs 736.5(151.8) μmol.l^{-1}, p=0.01). Also no correlation was found between plasma peroxidisability and TRAP [(tDC: r=0.008, p=0.972), (TBARS: r_s=0.184, p=0.390)]. These results suggest that diabetes *per se* is not associated with a lower TRAP but that an interaction may exist between diabetes and smoking which would help explain the even greater risk that smoking poses to diabetics and suggests that smoking diabetics would be more vulnerable to FR damage in circumstances of increased FR load such as following ischaemic episodes. Many explanations for the lack of correlation between peroxidisability and TRAP are discussed in the text. One possibility is that peroxidisability is infuenced by the balance between lipid soluble antioxidants and the availibility of oxidisable substrate and that TRAP is chiefly contributed to by water soluble antioxidants. Vitamin E is the major lipid soluble antioxidant and this balance can be represented by the ratio of the concentrations of vitamin E to cholesterol plus triglyceride termed the vitamin E status. A study was undertaken to test the hypothesis that:- individuals suffering from diabetes have lower vitamin E levels relative to levels of oxidisable lipids than do non-diabetics;

or in other words, have lower vitamin E status which is in turn associated with evidence of greater lipid peroxidation in diabetes.

Plasma vitamin E status and TBARS concentration were determined in thirty five diabetic and twenty nine non-diabetic subjects. Vitamin E status was not significantly different in the diabetic and non-diabetic subjects (5.19(1.22) vs 5.48(1.03) $\mu M.mM^{-1}$, $p=0.309$). TBARS concentrations were significantly higher in diabetics than non-diabetics (2.40(0.69) vs 1.67(0.63) $\mu molMDA.l^{-1}$, $p<0.001$) but did not correlate with vitamin E status. This study confirmed reports that increased lipid peroxidation occurs in diabetes but suggest that this is little influenced by vitamin E status.

Epidemiological evidence suggests an inverse correlation between dietary antioxidants and the risk of atherosclerotic disease. Ten diabetics and non-diabetics took part in a study aimed at testing the hypothesis that:- vitamin C and vitamin E dietary supplementation increase the TRAP and vitamin E status of plasma respectively, and reduces lipid peroxidation in diabetics and non-diabetics. In addition to the effect of vitamin supplementation upon plasma TBARS the effect on copper stimulated oxidation of LDL isolated by ultracentrifugation was also assesed. Vitamin C, 1g per day, was associated with a rise in TRAP, a reduction in vitamin E status and possibly with a delayed rise in TBARS. These findings could be explained by a pro-oxidant effect of high dose vitamin C in individuals on a normal diet containing iron. Vitamin E, 300mg per day, was associated with a rise in vitamin E status, no change in TRAP, no change in several parameters of LDL oxidation but with a fall in TBARS. Although the latter result is consistent with a reduction in FR lipid oxidation in response to vitamin E supplementation several other explanations are discussed in the text. The presence of diabetes did not alter the response to vitamin supplementation.

Overall the studies in this thesis do not provide evidence for the contention that diabetes might be a state of excess FR lipid oxidation thus explaning its associated high risk of atherosclerotic disease.

CHAPTER ONE

INTRODUCTION
LOW DENSITY LIPOPROTEIN OXIDATION IN ATHEROSCLEROSIS AND THE INFLUENCE OF DIABETES.

1.1. CARDIOVASCULAR DISEASE RISK FACTORS AND DIABETES

Atherosclerotic cardiovascular disease (ACVD) is the major cause of death in the western world. For unidentified reasons, individuals suffering from diabetes are at even greater risk of being affected than non-diabetics (1,2). In diabetes, the underlying pathology of atheroma arises earlier and more extensively than in non-diabetics (3). Thrombotic events which herald acute crises in the condition, such as myocardial infarction, occur more frequently in diabetic subjects, and the outcome of each event is more often fatal (4).

Many risk factors for atherosclerosis in the general population have been identified; the main factors being hyperlipidaemia, especially raised concentrations of low density lipoprotein (LDL), hypertension, smoking, age and male sex (5). The same risk factors are believed to operate in diabetes (6) except that the relative protection afforded to pre-menopausal women is lost in diabetic women (7). However, neither the fact that these risk factors do tend to cluster in diabetes (8) nor the fact that they may positively interact with diabetes (5,9) accounts for the excess risk associated with diabetes. Diabetes appears to be an independent risk factor (6).

Regardless of the combination of risk factors to which an individual is exposed, the underlying atheromatous pathology of cardiovascular disease is essentially the same. Similarly, histological studies of atheroma in diabetes have shown the pathology to be equivalent to that in non-diabetics (3). This suggests that the basic insults to the vessel walls are the same and that even if the different risk factors mark different causal mechanisms they may ultimately work through a common pathway.

The association of a factor with a disease is not proof of causality but any proposed theory for the development of atherosclerosis will hold up only if it is also able to explain the associations. Current opinion increasingly favours a mechanism involving free radical (FR) oxidation of low density lipoprotein (LDL) as an early

event in the development of atheroma (10), hence this mechanism provides an explanation for the observed associations between LDL and atherosclerosis. In this thesis I explore the possibility that this mechanism is enhanced in diabetes, thereby explaining the greater risk of atheroma seen in association with diabetes. I also discuss briefly the possible links between LDL oxidation and other cardiovascular risk factors and so, the ability of this theory to tie all the major associations together when viewed as a final common pathway for the generation of atherosclerosis.

1.2. THE LOW DENSITY LIPOPROTEIN OXIDATION THEORY OF ATHEROGENESIS

Many of the topics dealt with in this introduction will be expanded upon in later chapters but are discussed here to provide the background for the developement of the hypotheses explored in this thesis. Since free radical (FR) activity is an integral part of the proposed mechanism I will first summarise some relevant features of FR biology.

1.2.1. Free Radicals.

a. The Nature of Free Radicals

FRs are atoms or molecules in which one or more electronic orbital contains a single, unpaired, electron. This concept can be more easily appreciated with reference to a schematic model of atomic structure (Fig 1.1.). Orbitals have an associated energy level and electrons fill them in accordance with two basic principles. Firstly, electrons fill orbitals from the lowest energy level up. Secondly, the magnetic properties of spinning electrons mean that electrons must have opposite spins to occupy the same orbital. Hence, no more than two electrons can exist in a single orbital and they must be of opposite spin.

When atoms covalently bond to form molecules, two atomic orbitals interact to form two new molecular orbitals; one "bonding" orbital of lower energy, one "antibonding" orbital of higher energy (Fig 1.1b). In forming the molecule, the electrons rearrange but fill the new molecular orbitals obeying the same principles as above.

In the figure, oxygen is used as an example. The oxygen atom, having 8 electrons (atomic number 8), has orbitals filled as shown (Fig 1.1.a.). Since it has two

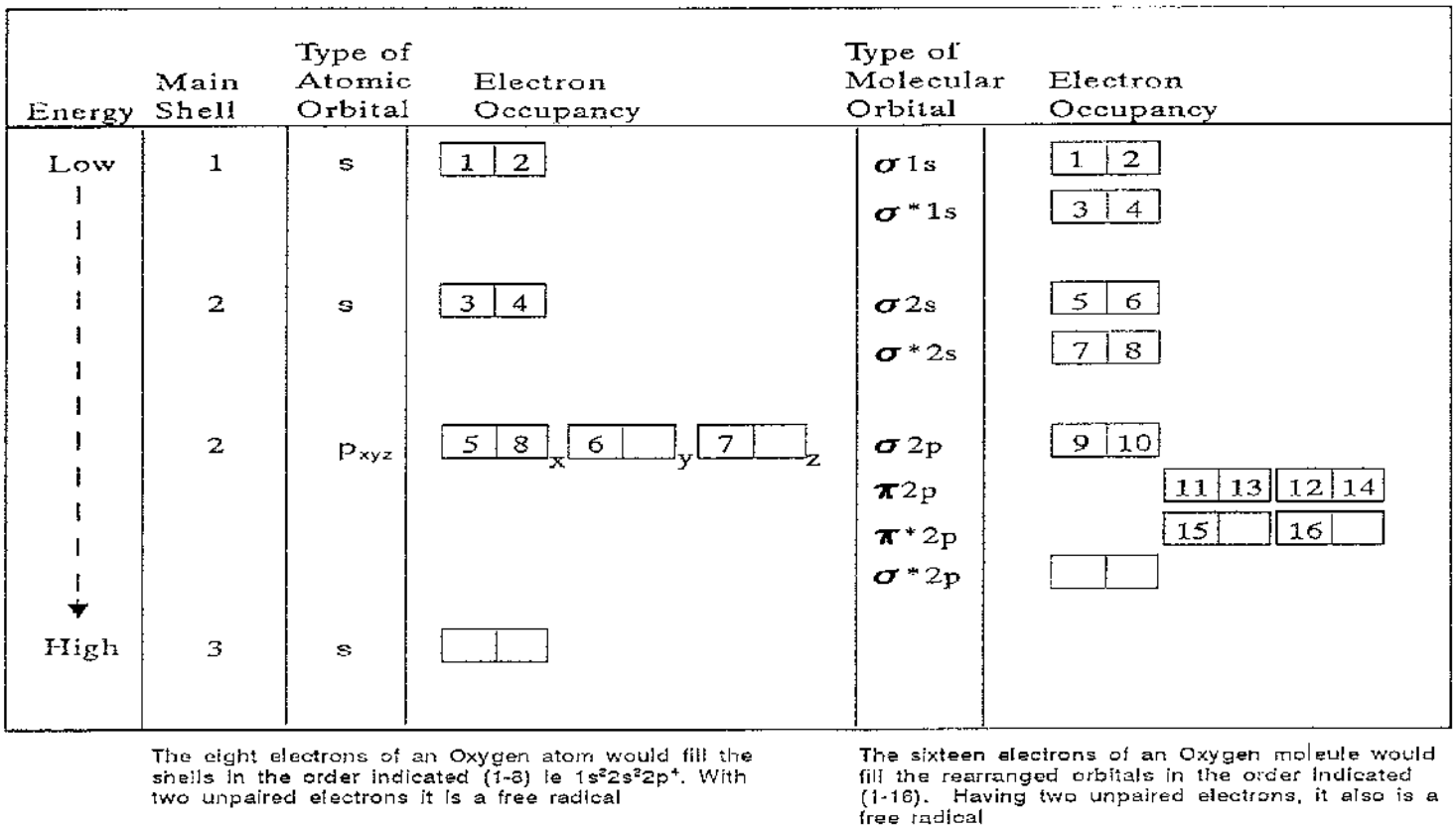


Figure 1.1: Atomic and Molecular Orbital arrangement for the two inner electron shells

unpaired electrons it is a radical. A more stable state is reached when the oxygen atom has a filled outer electron shell similar to that of neon. It achieves this state by sharing two electrons as in H_2O or O_2. In O_2, the 16 electrons fill the orbitals as indicated in Fig 1.1.b. Again there are two unpaired electrons so O_2, although more stable than O, is also a radical, in fact a biradical. If O_2 is reduced by the acceptance of a single electron it remains a radical, namely the superoxide anion. This is represented as $O_2^{\cdot-}$, the superscripted dot being the standard symbol indicating a free radical species. Two electron reduction of O_2 produces the non-radical peroxide ion (O_2^{2-}), however as the extra electron enters antibonding orbitals the 0-0 bonding is weakened helping to explain the reactivity of peroxide species. In biological systems, two electron reduction of O_2 usually results in hydrogen peroxide (H_2O_2) which can decompose to give two highly reactive hydroxyl radicals ($OH^{\cdot}$).

Because their electronic arrangement is unstable, FRs are, on the whole, highly reactive, although the degree of reactivity varies from species to species. They can achieve increased stability by losing or gaining an electron and so are capable of oxidation or reduction, and so of initiating, and taking part in, chain reactions (11).

b. Free Radical Sources *in vivo*.

One of the first processes to be identified as a FR chain reaction was the rancidification of fats *in vitro* which involves their oxidation and peroxidation (12). Subsequently the critical role of FRs in much of polymer chemistry was recognised and revolutionised industrial chemistry, nevertheless it was believed, until relatively recently, that such reactive species could not possibly be tolerated within living organisms and so would not exist *in vivo* (11).

It is now recognised, however, that FRs arise *in vivo* as essential intermediates in many metabolic pathways, especially those which involve cycles of oxidation and reduction and that intermediary metabolism might actually be regarded as making use of their reactive features. Perhaps the best example of such a pathway is the mitochondrial electron transport chain which may be the major source of FR *in vivo* (13). Single electrons are transferred from one species to another within the chain, finally reducing O_2 in a stepwise fashion to H_2O. Such single electron transfer inevitably means that FR intermediates are formed but in general they remain enzyme bound, for example O_2 and its partially reduced intermediates are bound to cytochrome oxidase. FR intermediates also arise during the enzymatic production

of prostaglandins, thromboxanes and leukotrienes. During these reactions oxygen is incorporated into polyunsaturated fatty acids (PUFA) (14,15) and the isolated enzymes, prostaglandin endoperoxide synthetase and lipoxygenase, have been shown capable of generating and releasing $O_2^{\cdot-}$ into the medium (16). The oxidation reactions controlled by cytochrome P_{450} (13) are another source of FR *in vivo*.

Since the FR intermediates in these pathways are tightly enzyme bound their further reactions are limited to those specified by the enzyme's activity. However, some of the electrons being transferred are believed to escape or leak from their intended path to react with any neighbouring molecule capable of acting as an electron acceptor, such molecules being termed oxidising agents, and such reactions inadvertently give rise to an unbound radical. Oxygen (O_2), a bi-radical, is freely available and is a willing acceptor of single electrons which, as described above, results in the formation of the more active superoxide radical ($O_2^{\cdot-}$) (Equation 1.1) (17). This radical can then react further giving rise to a family of reactive O_2 species including the non-radical hydrogen peroxide (H_2O_2) (Equation 1.2) and the very active hydroxyl radical ($OH^{\cdot}$) (Equation 1.3). This formation of $OH^{\cdot}$, by what is called the Haber-Weiss reaction (Equation 1.3), would occur so slowly as to be biologically irrelevant, however, this reaction is greatly catalysed by transition metals, especially ferrous iron (Fe^{2+}).

Equation 1.1 $\quad O_2 + e^- \rightarrow O_2^{\cdot-}$

Equation 1.2 $\quad 2H^+ + O_2^{\cdot-} + O_2^{\cdot-} \rightarrow H_2O_2 + O_2$

Equation 1.3 $\quad H_2O_2 + O_2^{\cdot-} \rightarrow OH^{\cdot} + OH^- + O_2$

Transition metal ions are actually FRs and the ease with which they change valency allows them to catalyse the formation and interconvertion of these reactive oxygen species and gives them a central role in FR biology (Fig 1.2) (17). In addition to reactions with H_2O_2, transition metals can react with other organic peroxides, giving rise to a series of oxygen centered radicals (Fig 1.2) The binding of transition metals in protein complexes such as transferrin and caeruloplasmin may partially, but not completely, restrict this catalytic activity (15). FR are also produced *in vivo* during the spontaneous oxidation of molecules such as haemoglobin, catecholamines and even glucose itself (13,18).

In addition to their existence as both useful intermediates and inadvertent by-products of enzymatic and non-enzymatic reactions, FR's are also the intended end

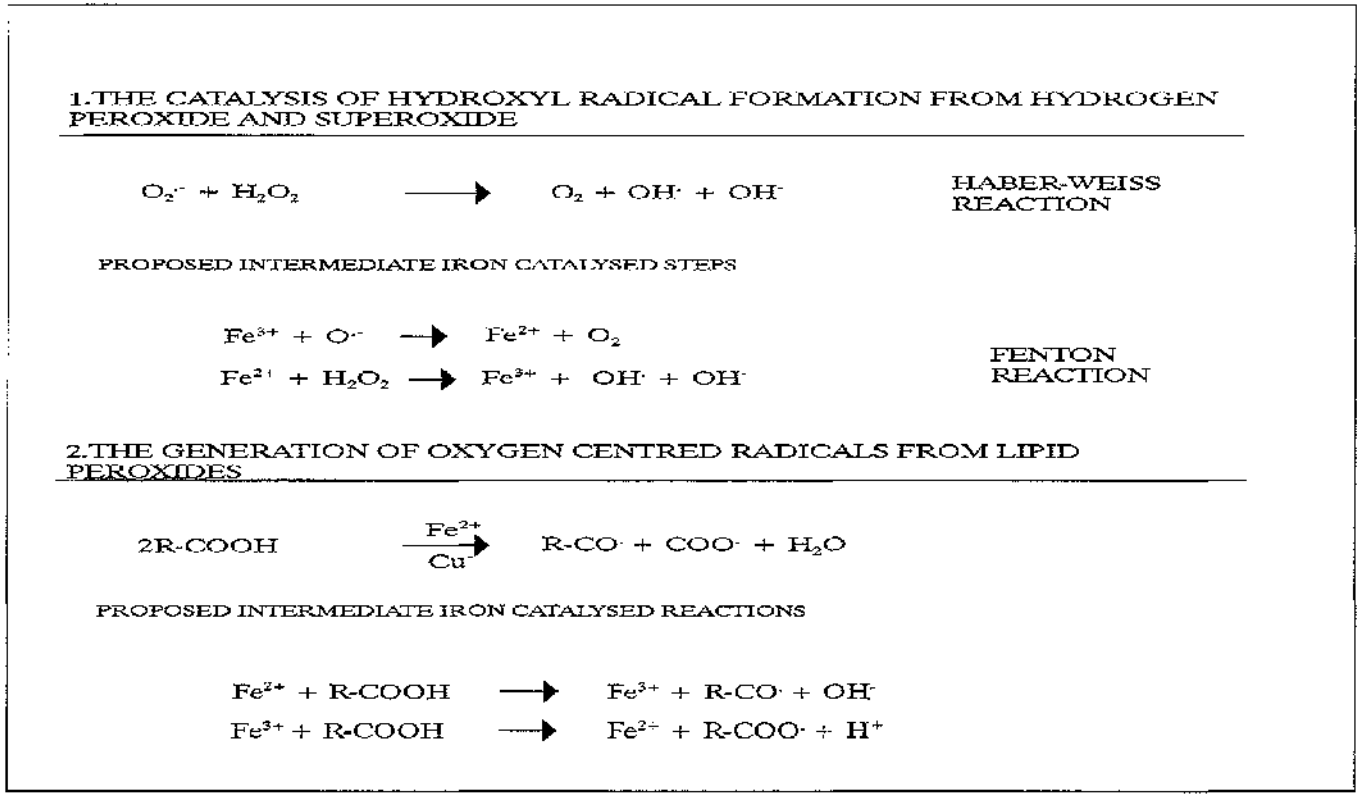


Figure 1.2: The role of transition metals as catalysts in radical reactions

product of a few reactions thus neutrophil NADPH Oxidase produces a superoxide radical to take part in bacterial killing (19). However even when production is intended, inappropriate or excess release of FR by activated neutrophils may add to the tissue damage associated with infective and inflammatory processes (19,20).
A pathological situation in which increased FR production is believed to occur, and then contribute to tissue damage, arises in association with ischaemia. There is increasing evidence that during periods of tissue ischaemia changes occur which allow for the formation of reactive oxygen species when O_2 is reintroduced to the system. These changes may include release of iron from storage proteins, altered substrate specificity of Xanthine Oxidase, and a build up of hypoxanthine. When O_2 is returned to the system, hypoxanthine is metabolised by Xanthine Oxidase, resulting in $O_2^{\cdot-}$ production (21,22,23).

c. Free Radical Defensive Mechanisms

Inevitably such reactive species are potentially hazardous and for FRs not generated in a controlled, restricted environment, protective mechanisms have evolved. Indeed, perhaps as evidence of their potential escape, and biology's perception of them as a possible threat, many aerobic organisms have been found to have an enzyme, Superoxide Dismutase (SOD) (24), with no apparent role other than removal of $O_2^{\cdot-}$ which is done by catalysing its dismutation to H_2O_2 (Equation 1.4).

Equation 1.4 $$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

H_2O_2 is not an innocuous substance and when generated in this, and other reactions, it is in turn removed by Glutathione Peroxidase and Catalase (Fig 1.3.) (17). Selenium (Se) is necessary for the activity of Glutathione Peroxidase and glutathione (GSH) is a co-substrate in this reaction being oxidised, and so temporarily inactivated, during this process. GSH is then reduced and reactivated by a NADPH-dependent reductase. NADPH is thus the ultimate antioxidant in this series of recycling reactions (Fig 1.3) and its availability is determined by the balance between the activity of processes during which it is produced and during which it is utilised. The pentose phosphate pathway is an important source of NADPH. The polyol pathway, a pathway believed to be more active in diabetes, is among many, a net user of NADPH (25). The significance of an imbalance in these pathways in terms of antioxidant reserve in diabetes is discussed further in section 1.3.2.

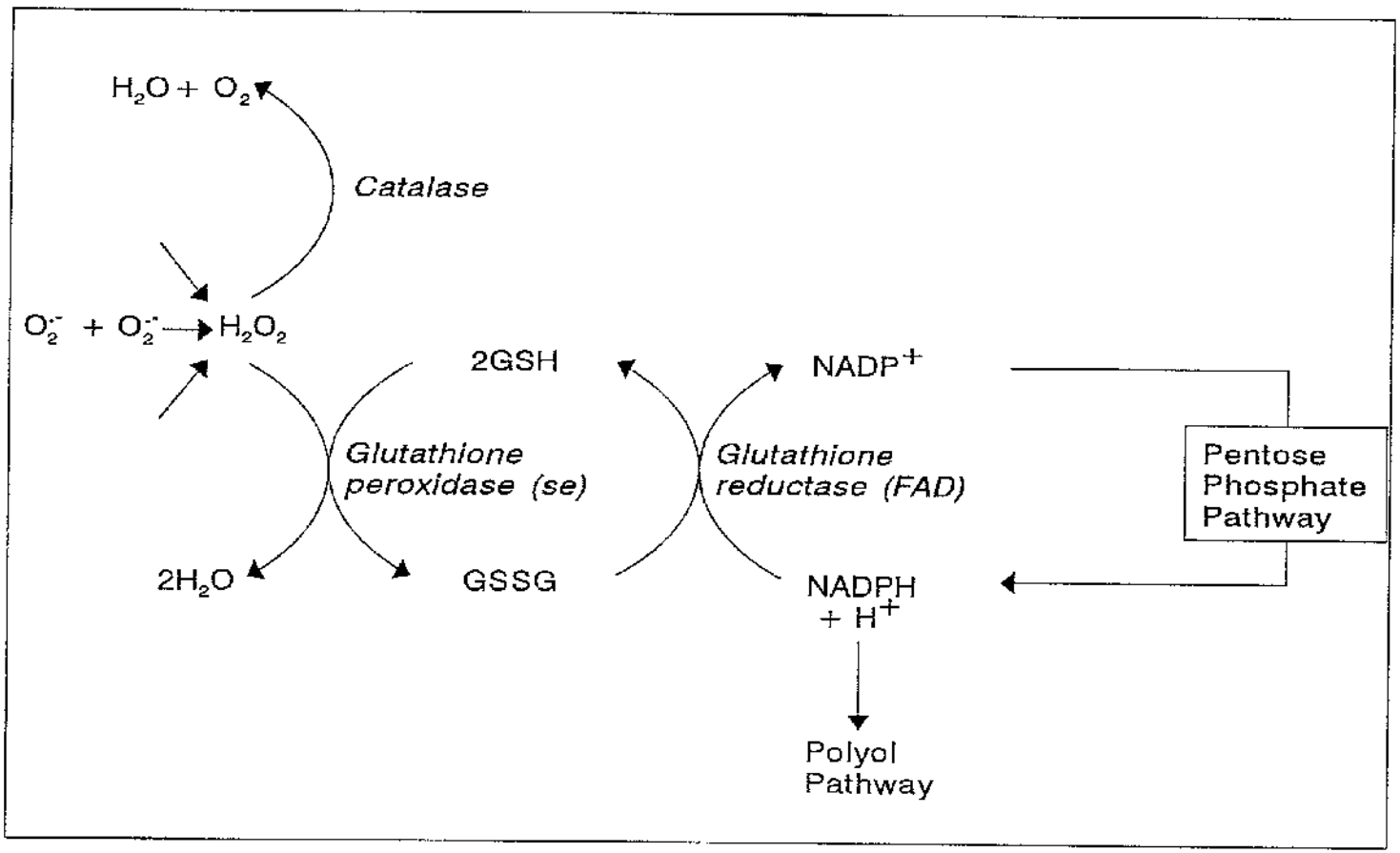


Figure 1.3: Redox recycling reactions involved in the removal of hydrogen peroxide.

In addition to these enzymatic defenses, non-enzymatic protective mechanisms against FR activity are also available. Exogenous species such as the vitamins E, C, and A, and ß-carotene, and endogenously generated molecules such as uric acid, bilirubin and the thiol groups on proteins, can act as antioxidants reacting with and inactivating FRs (26-30). Many of these species have roles in metabolism in addition to that of antioxidant however no other role has been identified for vitamin E.

d. Consequences Of Unwanted Free Radical Activity

Should all of these defensive mechanisms be overwhelmed FRs would theoretically be capable of reacting with endogenous lipids, proteins, nucleic acids and carbohydrates thus altering their ability to function. As frequently reviewed, the potential consequences are legion; from changes in membrane fluidity and function, to effects on cell division and repair mechanisms (11,15,17). As a result, FR activity has been implicated in many disease processes, such as rheumatoid arthritis, carcinogenesis, emphysema and including atherosclerosis. The consequences of FR attack on lipids, and in particular lipids within LDL, are of most immediate interest in this thesis and are dealt with in more detail in chapter three. Such attack on the most vulnerable of the lipid classes, namely the polyunsaturated fatty acids (PUFA), gives rise to several detectable oxidation products (Fig 3.1). Diene conjugated fatty acids (DCFAs), produced by changes in electronic structure, have a typical UV absorption peak, allowing their spectrophometric detection (31). Other changes in chemical structure give rise to species such as hyproperoxides and malondialdehyde, detectable by their reaction with thiobarbituric acid (thiobarbituric acid reactive substances (TBARS)) (32). Both DCFAs and TBARS are detectable in biological samples,implying that these reactions also occur *in vivo*, and so concentrations of these compounds are used as markers of FR activity *in vivo* (Fig 3.1).

1.2.2. Modification Of Low Density Lipoproteins And Foam Cell Generation

Having described some of the salient features of FRs, I will now discuss their role in LDL oxidation and its relevance to atherosclerosis.

a. Formation Of Foam Cells.

Epidemiological and pathological evidence has long implicated low density

lipoprotein (LDL) as pivotal in the generation of atheroma (33). However, until recently, the mechanisms proposed to explain the connection have not been persuasive. An early pathological feature of atheroma is the foam cell. Foam cells are mainly monocyte-derived macrophages laden with cytoplasmic lipid droplets (34). The lipid present in a foam cell is derived from plasma LDL (35). Macrophages have specific receptors for LDL which recognise sites on the apoprotein B component of the LDL particle and are referred to as the Apo-B 100 receptors. Macrophage cholesterol content is an equilibrium between cholesterol uptake from LDL bound by this receptor, its *de novo* synthesis and its release from cells. This balance is normally maintained by down regulation (negative feedback) on the receptor and on cholesterol synthesis when the cell is replete with cholesterol (36). Thus this method of lipid uptake by macrophages could not explain the overloading required to generate foam cells. In keeping with this observation, macrophages cannot be converted to foam cells *in vitro* even when exposed to very high concentrations of LDL (37). In addition, individuals suffering from familial hypercholesterolaemia, who have a hereditary lack of functional classical LDL receptors (38), nevertheless do form foam cells which contain LDL derived cholesterol and are subject to even greater risk of atherosclerosis than the general population (39). Therefore, the lipid loading of macrophages *in vivo* inferred the involvement of pathways other than the classical pathway for LDL uptake.

b. Modification Of Low Density Lipoprotein.

This possibility of alternative uptake pathways of LDL-cholesterol was supported when a chemical modification of LDL *in vitro*, namely acetylation of Apo-B ϵ-amino groups, was noted to result in accelerated macrophage LDL uptake and foam cell formation (37). This uptake was saturable and not inhibited by native LDL, implying the involvement of a receptor distinct from the Apo-B 100 LDL receptor. This new receptor was called the acetyl or scavenger receptor. Other modifications of LDL have subsequently been found which allow uptake by this pathway (40). It is now also recognised that more than one scavenger receptor exists (41). Uptake by these receptors is not subject to down regulation, and hence would enable foam cell formation *in vivo* if LDL modification occurred *in vivo*. The discovery that LDL incubated with endothelial cells, smooth muscle cells or fibroblasts, could result in a modification which also allowed uptake via scavenger receptors made *in vivo* modification more probable (42,43).

Cell mediated modification is not, however, a simple protein acetylation. The modified LDL formed has been characterised and is chemically, physically and functionally different from native LDL (10,43,44). Among its many chemical differences it contains oxidised derivatives of the lipid component including lipid hydroperoxides (45). As mentioned earlier, lipid oxidation is a FR, peroxidative, process *in vitro* (11). There is now a large body of evidence suggesting that cell mediated LDL modification also intimately involves, and indeed is initiated by, FR oxidation of the lipid component. Transition metals catalyse FR lipid oxidation and are necessary in trace amounts for cell mediated modification (45) also if LDL is exposed to Cu^{2+} or Fe^{2+} in a cell free system the modified LDL which results has similar characteristics to cell modified LDL (45,46). Other conditions associated with FR generation, such as steady state radiolysis, also result in LDL oxidation and an LDL modification similar to that induced by cells (47). Also it has been found that the ability of cells to modify LDL related to their ability to generate $O_2^{\cdot-}$ and that FR scavengers and superoxide dismutase inhibit cell mediated modification of LDL (43,44). All of these findings suggest that cell mediated modification is also a free radically mediated oxidative process. The alterations in the protein component of LDL are believed to be secondary to this lipid oxidation in that the lipid peroxide products ultimately breakdown to a series of aldehydes which react with and lead to derivitization of the protein lysine residues (48). It may be that these secondary changes in the protein component of LDL which alters it's receptor interactions and so macrophage uptake. Apoprotein B is also fragmented in the modification process, with reduction of its lysine, histidine and proline content (47,48). In addition to acting as a possible source of FRs the cells capable of oxidising LDL may do so *via* a second mechanism. Cellular lipoxygenase activity has been shown to have a possible role in endothelial cell oxygenation of LDL and it may be that lipids enzymatically oxidised within cells are transferred to LDL where they can then furter react in the presence of transition metals (49). A phospholipase A_2, cleaving oxidised lipids from the second carbon of phosphatidylcholine, also has been shown to be involved in the cellular modifacation of LDL but the exact role that it plays is not yet clear (10,45,50).

c. Consequences Of Low Density Lipoprotein Modification.

The oxidative chemical changes of LDL are associated with typical physical changes in density and charge, and with many functional changes. In addition to the altered

uptake of oxidised LDL by macrophages which allows foam cell formation (42), an ever increasing number of other activities theoretically of importance to atherogenesis are also being ascribed to oxidised LDL. For example it has been found to be immunogenic (51), chemotactic for monocytes, and cytotoxic to endothelial, smooth muscle cells and fibroblasts (52). In addition the lipid peroxides in which it is enriched may alter eicosanoid metabolism, and so haemostatic and fibrinolytic mechanisms (53). Since platelets appear to have a role in the development of atherosclerosis and their aggregation is closely linked to eicosanoid metabolism (54) any ability of oxidised lipids to modify eicosanoid metabolism raises the possibility that alteration in platelet function might be another mechanism whereby oxidised lipids could enhance atherosclerosis. Furthermore fibrinolysis may also be impaired by the endothelial cell synthesis of plasminogen activator inhibitor (PAI) which has been shown to be stimulated by oxidised LDL (55). Other endothelial cell functions, such as endothelial cell induced vascular relaxation, may also be altered by oxidised LDL (56,57).

d. Oxidised Low Density Lipoprotein *in vivo*.

Although several cell types can induce modifications of LDL capable of supporting foam cell generation *in vitro* it cannot be assumed that such reactions occur *in vivo*. However, in support of this possibility is the finding of evidence indicating the existence of oxidised LDL *in vivo*. Autoantibodies to oxidised LDL have been found in human serum (51,58). Moreover, antibodies raised to oxidised LDL stain rabbit aortic atherosclerotic lesions (51,59) and oxidised LDL can be eluted from such lesions (51). In addition, LDL gently extracted from human and rabbit atherosclerotic plaques was found to have density, electrophoretic mobility and alterations in cholesterol and apo-B comparable to those reported for *in vitro* oxidised LDL (60,61). Also, lesion LDL and oxidised LDL were taken up and degraded by macrophages using the scavenger receptor whereas native LDL was not and lesion LDL was chemotactic for macrophages (60).

The oxidised LDL discussed so far is a highly modified form of LDL, explaining its marked functional difference from native LDL and the evidence for its existence in atherosclerotic lesions is as cited. However, this highly modified species is not found circulating in plasma and current opinion is that it is probably only formed extravascularly (9). On the contrary the term "minimally modified LDL" has been adopted to describe an oxidised LDL which is found in plasma and which contains

small, but detectable, concentrations of TBARS, but in which the apo-B is not modified. Minimally modified LDL is thus still recognised by the apoB-100 receptor (62), is not immunologically different from native LDL and does not support foam cell generation. Nevertheless, minimally modified LDL may be important in the even earlier stages of atherogenesis, as it has been shown to induce endothelial cells to produce macrophage binding, chemotactic and colony stimulating factors (63,64). Minimally modified LDL could then be responsible for the initial movement of macrophages into the intima. Once there, macrophages would contribute to the further oxidation of extruded LDL and eventually, foam cell generation takes place. Further progression of the early fatty streak to advanced atherosclerotic lesions may be enhanced by the cytotoxic and immunogenic features of highly oxidised LDL (9). The source of minimally oxidised LDL is not established but dietary lipids are oxidised to varying degrees and so diet may be one source.

1.3. LOW DENSITY LIPOPROTEIN OXIDATION IN DIABETES.

As outlined in the previous section, FR oxidation of LDL does seem an attractive mechanism to explain both the development of the foam cell and the association between LDL and atheroma, but can it explain the associations with the other risk factors, particularly diabetes? Is there any reason, or evidence, to suspect that more undesirable LDL oxidation might occur in diabetes? Using the indirect approach of measuring concentrations of products of FR attack on endogenous molecules, some evidence of excess oxidation in diabetes does exist. Lipid peroxides are one of the products of attack on lipids and are most frequently measured as TBARS. Plasma TBARS have been reported to be higher in diabetic subjects than controls by many groups (65-67). However, there is some debate as to the independence of diabetes in producing this effect since it is also reported that diabetic subjects without angiopathy had plasma TBARS not significantly different from controls (67). Also, Velàzquez *et al* reported no independent correlation of TBARS with measures of glycaemia (67). On the other hand, Jain *et al* found that in a group of diabetic children erythrocyte membranes had a higher TBARS content compared to controls and that TBARS content did correlate with HbA_{1c} (68). Other diabetic tissues have also been found to have increased TBARS. Cataractous lenses from diabetic subjects

were found to have greater TBARS than lenses bearing senile cataracts or clear lenses (69).

Another consequence of FR attack on lipids is the formation of diene conjugated fatty acid species (DCFA). These have also been reported to be elevated in diabetics with angiopathy (70). However, Collier *et al* reported reduced DCFA in a group of uncomplicated insulin-dependant diabetic subjects (71). This contradiction may be partially explained by the possibility that DCFAs within tissues do not arise solely as a result of FR activity (72). DCFAs exist in dietary compounds and so diet may be a confounding source. Products of FR attack on molecules other than lipids, such as proteins (73), are less well characterised, however, it is possible that the changes in proteins seen in diabetes, and attributed solely to glycosylation, partially reflect such attack (74, 75). Such protein changes may be further evidence of greater FR oxidative damage in diabetes (75).

If, as is suggested, these differences mean that more FR oxidation does occur in diabetes it begs the question why? More oxidation could occur in circumstances of :-

(1) more FR generation,

(2) reduced availability of protective mechanisms or

(3) greater availability of oxidisable substrate.

I would like to discuss each of these possibilities in turn.

1.3.1. Free Radical Generation In Diabetes

In very general terms diabetes can be considered a disorder of intermediary metabolism resulting from impaired action of insulin. The basis of this impairment may not be the same in the two major types of diabetes. Insulin deficiency is accepted as the basic abnormality in Type 1 diabetes but it is currently debated whether absolute or relative insulin deficiency and/or insulin resistance with hyperinsulinaemia is the underlying problem in Type 2 diabetes (76-79). Part of the difficulty in fesolving this debate has arisen because of the lack of specificity of older insulin assays but with the advent of newer more specific techniques this issue may soon become clearer (79,80). Nevertheless, individuals suffering from both types of diabetes are at greater risk of atherosclerosis than non-diabetics (81, 82). In Type 1 diabetes the increased risk of mortality from coronary heart disease is most marked in those subjects with nephropathy (83, 84). However, those without

nephropathy have also been shown to experience a greater risk than the non-diabetic population (83). The coronary heart disease mortality rate in Type 2 diabetic subjects has been estimated as 2 to 4 times greater than that in non-diabetic subjects (2). Since both types of diabetes are associated with coronary heart disease it is reasonable to look for factors common to both to explain the association. Even if abnormalities of insulin concentration *per se* may not be common to both types of diabetes impaired insulin action, and some of its metabolic consequences, is. In both types of diabetes, whether the problem is insulin deficiency or resistance, impaired insulin action is accompanied by major alterations in many metabolites, including the pathognomonic rise in plasma glucose and the consequent excess of non-enzymatic glycosylation of structurally and functionally important macromolecules (85, 86). It has always been tempting to try to ascribe the complications of diabetes to these metabolic alterations, especially to hyperglycaemia as the most prominent and universal abnormality. Microvascular complications do seem to be closely associated with hyperglycaemia (87) but the picture is much less clear when considering atherosclerosis. As I will describe below, I would like to speculate that looked at from a FR point of view the links between hyperglycaemia, and some other fundamental metabolic abnormalities of diabetes, and atherosclerosis might be more clearly seen and this approach also allows for the fact that both types of diabetes are associated with atherosclerosis.

The ultimate aim of intermediary metabolism is to provide the energy for all functions necessary to maintain life. High energy phosphate bonds, predominantly in adenosine triphosphate (ATP), but also in guanosine triphosphate (GTP), are the immediate source of that energy. Their generation is a basic function of intermediary metabolism and is coupled mainly to reduction of O_2 in the mitochondrial electron transport chain, in turn powered by the oxidation of organic fuels in the form of carbohydrates, fats and proteins. Theoretically, the stoichiometry of this overall reaction can be determined and is such that the most efficient generation of ATP comes about when glucose is oxidised via glycolysis and the citric acid cycle. When this is the case 3 moles of ATP are produced for every mole of oxygen reduced. When fats are oxidised this number is reduced to 2.8 moles of ATP per mole of O_2 (88). Another way of expressing this is in terms of the energy produced per litre of O_2 consumed whilst oxidising different fuels. Indirect calorimetry shows again that more energy is derived per litre of O_2 when oxidising carbohydrate than when oxidising fats (89). The consequence is that,

should circumstances arise in which relatively more fat oxidation occurs in order to maintain energy supplies, then, more electrons would have to move through the electron transport chain. Since this chain is possibly the major source of FR production, increased fat oxidation might result in increased FR load. Because FRs initiate chain reactions, only a relatively small change in absolute FR production would have disproportionately large consequences. There is evidence that more fat oxidation does occur in both types of diabetes. In hyperglycaemic, non-obese, non-insulin dependent diabetic subjects lipid oxidation was found to be greater than in controls using indirect calorimetry (90). In poorly controlled insulin dependent diabetes excess oxidation of fat is a classic feature (25). Why fat oxidation is increased in diabetes may result from several mechanisms and is closely linked to glucose metabolism. In fact, carbohydrate and fat oxidation are regulated such that effects upon one are frequently associated with reciprocal effects on the other. Insulin stimulates several of the steps involved in glucose oxidation but inhibits oxidation of fat (25, 91). Also, operation of the Randle cycle means that greater oxidation of fat is associated with reduced carbohydrate oxidation and vice versa (92). This cycle would allow a vicious circle of falling carbohydrate oxidation and rising lipid oxidation to arise. Such reciprocal regulation may help to explain the inverse relationship between glucose and lipid oxidation reported in non-obese NIDDM subjects (93, 94). In addition to reports of increased fat oxidation, it is also reported that glucose oxidation is reduced in diabetes (94, 95). Although it is recognised that the development of hyperglycaemia, by a mass action effect, may return glucose oxidation towards normal in NIDDM (93, 96), it has also been reported that even in the presence of hyperglycaemia, glucose oxidation remains impaired (94). Hence, there is evidence that energy production in both types of diabetes may be associated with a shift in the fuels used, with more fat oxidation and less glucose oxidation (97, 98).

Assuming that diabetic subjects require at least the same amount of ATP as their non-diabetic counterparts, and if they are oxidising more fat to obtain it, then, as argued above, this may be one source of excess FR load in diabetes. The situation may even be further exacerbated as their is evidence that diabetes is associated with increased resting energy expenditure, and so ATP requirements, perhaps because ATP is consumed during gluconeogenesis which occurs to excess in diabetes (90, 99).

Another of the basic features of diabetes is non-enzymatic glycosylation of

macromolecules. The initially reversible product of the reaction between glucose and protein lysine groups undergoes many rearrangements via a stable Amadori product to a denatured, fragmented macromolecule which can not function normally and contains cross-links and fluorescent structures. The exact nature of these final products is not clearly known but they are referred to as advanced glycosylation end products (AGE) (86). It was felt that these chemical and functional changes were the direct consequence of glycosylation and might be involved in the pathophysiology of the complications of diabetes (85, 86, 100). However, the end products thought typical of protein glycosylation can also be produced by FR attack upon proteins (73) and more recently it has been realised that glycosylation may actually involve FR mechanisms (75, 101).

Glucose oxidises in the presence of O_2 and transition metals to a ketoaldehyde accompanied by the production of H_2O_2 (74) and $OH^{\cdot}$ (18, 102). During experiments in which albumin was incubated with glucose it was found that when glucose oxidation was inhibited, by chelating agents such as diethylenetriaminepenta-acetic acid (DETAPAC), then glucose incorporation into albumin was reduced although not completely inhibited. Also it was found that preformed ketoaldehyde could react with protein, leading to glucose incorporation (73). These findings suggested that more than one mechanism may be involved in glycosylation and that some glycosylation results from the direct reaction of glucose with protein whilst a proportion of the glycosylation which occurs requires the oxidation of glucose and then the reaction of the resulting ketoaldehyde with protein. Consistent with such a theory is the finding that glycosylated haemoglobin formation is reduced by the presence of antioxidants (103). In addition, when $OH^{\cdot}$ radicals, also a product of glucose oxidation, were scavenged by sorbitol, then glycosylation was found to occur but the development of conformational changes and protein fragmentation did not. This suggested that glycosylation could be dissociated from these other changes and that they are not a direct consequence of glycosylation but involve further reactions which require the presence of $OH^{\cdot}$ (18). Hence, glucose oxidation provides a ketoaldehyde, which contributes to glycosylation, and FRs, which induce the fragmentation and conformational changes in proteins exposed to glucose. There is also evidence that the early products of glucose and ketoaldehyde linkage to proteins go on to further oxidise, with formation of yet more FRs capable of contributing to the damage done (18, 104). In keeping with these findings glycosylated proteins have been shown capable of $O_2^{\cdot-}$ production (105), and this

may explain the reported greater production of that ion by diabetic plasma (106). Singer *et al* argue that the lack of association seen over the years between atherosclerosis and plasma glucose is due in part to inadequate measures of glycaemia. This seemed consistent with their finding that when glycosylated haemoglobin (HbA_{1c}), believed to be a good measure of overall long term glycaemia was used, an association between the prevalence of cardiovascular disease in the female survivors of the original Framingham study cohort and HbA_{1c} was found (107). HbA_{1c} is a measure of glycaemia but if it's formation is also determined by FR mechanisms, as suggested above,then perhaps HbA_{1c} relates better to atheromatous complications because it is a "marker" of two of the processes important in the development of those complications or of the one process directly involved.

As already mentioned, eicosanoid metabolism may be another source of FRs *in vivo* and is also perturbed in diabetes (54, 108-110). Eicosanoids may influence atherogenesis in several ways (111, 112), one mechanism may operate through platelet aggregation. Platelet aggregation is controlled by platelet and endothelial eicosanoids so that diabetes related alterations in eicosanoid metabolism offers one possible explanation for the enhanced platelet aggregation found in diabetes (110, 113). Platelet aggregation *per se* has been attributed a role in the generation of atherosclerosis however, I would like to speculate that in addition the increased thromboxane production reported in diabetic platelets means increased production and release of $O_2^{\cdot-}$ with its attendant implications for atherosclerosis and this possibility is discussed further in chapter four. Such an interplay between $O_2^{\cdot-}$ release and eicosanoid formation has been suggested by work on glomerular monocyte/macrophages isolated from nephritic rat kidneys (114).

Yet another source of FR is the respiratory burst of activated neutrophils, and it has been shown that mononuclear cells from hypertriglyceridaemic diabetic subjects produce more superoxide than those from controls (115) although no explaination for this phenomenon was found.

1.3.2. Free Radical Protective Mechanisms In Diabetes

In addition to excess generation of FR species, a second situation in which more FR oxidation of LDL could occur is where protective mechanisms are reduced. In diabetes, along with less efficient glucose utilization in insulin sensitive processes,

by a mass action effect in the face of hyperglycaemia more glucose is shunted through alternative pathways and this can have secondary effects on FR activity via the protective mechanisms. For example, excess flux of glucose through the polyol pathway occurs in diabetes (116, 117). Aldose reductase is the rate limiting enzyme in this pathway and converts glucose to sorbitol using NADPH as reductant (25, 116). Hence the over-activity of this pathway in diabetes results in greater consumption of NADPH (116, 117). As already indicated, NADPH is necessary for the reduction of glutathione and thus for its reactivation as an antioxidant (Fig 1.3). Thus the normal redox recycling of antioxidants may be deficient in diabetes exposing subjects to the risk of FR damage even in the face of unaltered FR generation.

Reduced plasma levels of vitamin C have been reported in diabetic humans (119, 120) and rats (121, 122), as have reductions in the levels of protein thiol groups and the protective enzyme Superoxide Dismutase (123, 124). Karpen reported reduced platelet levels of vitamin E (125) and Chari reduced glutathione concentration in polymorphonuclear leucocytes (126). However, measurement of only one, or a few, of these protective factors is not without its problems. One of these problems is that interaction and synergy between the factors may mean that they can compensate for each other's deficiencies, so that an overall measure of antioxidant activity would be more informative. Wayner *et al* (127) described such an assay and determined the total peroxyl radical antioxidant activity of plasma (TRAP). The TRAP assay measures the ability of plasma,and its contained antioxidants, to inhibit attack on linoleic acid by peroxyl radicals, produced by the thermal decomposition of a known radical source, 2,2'-azo-bis(2 amidinopropane) hydrochloride (ABAP). The TRAP assay is discussed further later and has been used in several of the studies presented in this thesis.

1.3.3. Oxidisability Of Low Density Lipoprotein In Diabetes

In addition to excess FR production and impaired protective mechanisms, a third situation in which excess FR damage to LDL might be expected to occur in diabetes would be the presence of greater quantities of LDL or the appearance of LDL altered in such a way as to make it more easily oxidised. LDL particles are a composite of proteins, lipids and antioxidants (128). Alterations in any of these components could enhance the particles' oxidisability. Some alterations have already

been observed in diabetes and are discussed below, but their effects on oxidisabilty await exploration.

Dyslipidaemias do occur in diabetes and differ depending on the sex of the patient, the type of diabetes and the degree of control. However, LDL levels are not typically abnormal, diabetes being more commonly associated with hypertriglyceridaemia and low HDL concentrations (82). Therefore, simply increased availability of LDL would not offer an explanation for the possibility of enhanced LDL oxidation in diabetes.However glycosylation of LDL apoproteins has been reported in diabetes (129) and, bearing in mind the possible links between glycosylation and oxidation, may enhance LDL oxidation. Consistent with this idea exposure of LDL to glucose *in vitro* resulted in the appearance of lipid peroxidation products (102, 130), this being inhibited in the presence of DETAPAC (102).

Apart from possibly increasing the risk of oxidation of LDL, glycosylation of LDL *per se* has been reported to have other consequences of relevance to atherosclerosis (131). Whilst attempting to dissociate glycosylation from oxidation others report that glycosylated LDL enhances macrophage accumulation of cholestyl ester in its own right (131, 132). A receptor distinct from the classical LDL receptor and the scavenger receptor may be involved. Also, glycosylated LDL alters platelet and endothelial eicosanoid metabolism in ways which are believed to increase atherosclerotic risk in that platelet thromboxane production was increased and endothelial cell production of prostacyclin reduced (133). Glycosylation of LDL also seemed to be responsible for the greater ability of diabetic LDL to stimulate platelet aggregation (134).

Apart from glycosylation, diabetic LDL has also been shown to differ compositionally in several ways from non-diabetic LDL. One aspect of LDL composition which is altered is the ratio of lipid to apoprotein content (135, 136). These ratios help to determine the particle size and density and it has been shown in non-diabetic populations that small, dense LDL particles are associated with increased risk of myocardial infarction (137). Small, dense LDL particles have been reported in both type 1 and type 2 diabetes (138, 139). The reason why such particles might be associated with increased risk of atherosclerosis is unknown but Austin *et al* (137) argues that they are not an independent risk factor and merely mark other atherogenic features such as hypertriglyceridaemia and low HDL. The effect of LDL particle size and density distribution upon LDL oxidisability is debated (140,141). Studying the susceptability of isolated fractions of LDL to

copper stimulated oxidation groups such as that of Rengström *et al* (140) have suggested that size and density are not important determinants of oxidisability but found an association between LDL triglyceride content and the susceptibility to oxidation. Interestingly, there are reports of triglyceride enrichment of LDL in diabetes (136, 138, 139), and in both diabetics and non-diabetics with coronary heart disease (142). However, other groups, for example Tribble *et al* (141) have reported that the susceptibility of LDL to oxidation does increase with increasing density of the LDL particle. There are several differences in the conduct of these studies by Rengström and Tribble which might explain their apparently contradicting results. One difference is that although both investigate the copper induced oxidation of isolated LDL, they measure different parameters to assess susceptibility to oxidation. One measures the lag time before oxidation starts (140), the other measures time taken to reach half maximal oxidation (141). Which of these parameters is the most important pathophysiologically is not known and they may be governed by different features of LDL. This problem is discussed further in sections 6.6.2 and 8.1.1. Also, in the Rengström study, LDL was provided by men with coronary artery disease and separated into only two fractions, whereas Tribble isolated LDL from healthy controls and separated it into six fractions.

Vitamin E and other lipid soluble antioxidants are also an integral component of LDL particles (46, 143, 144). If antioxidant content were lower relative to oxidisable substrate, such as polyunsaturated fatty acids and cholesterol, this might be expected to expose LDL to increased risk of oxidation. The appropriateness of vitamin E levels in diabetes is not clear. Karpen *et al* (109) found plasma vitamin E levels to be normal in diabetics but did not correct for the elevated levels of lipids in their subjects and it is now recognised that vitamin E levels are really only interpretable in the light of plasma lipids (145). As noted earlier, vitamin E levels relative to platelet number were found to be lower in diabetics (125). The appropriateness of vitamin E concentrations in diabetics is dealt with further in chapter seven and it's effectiveness in protecting against lipid oxidation discussed in chapter eight.

The last component of LDL particles I want to discuss in terms of oxidation are the lipids themselves. One of the major types of lipid in LDL are the fatty acids esterified within cholesterol esters, triglycerides and phospholipids. Of these, the polyunsaturated fatty acids (PUFAs) are more vulnerable to FR attack than monounsaturated (MFAs) or saturated fatty acids (SFAs) (146). A rise in the

PUFA:SFA ratio of LDL might, therefore, be expected to increase its oxidisability and vice versa. This seemed to be confirmed by the finding that enriching LDL with oleic acid, a MFA, and reducing its content of linoleic, a PUFA, resulted in resistance to oxidation *in vitro* (146). Relatively little is known about the PUFA:SFA ratio of serum lipid in diabetes but, since fatty acid metabolism is abnormal in diabetes (128, 147), it would not be surprising if the ratio were perturbed. The information actually available is conflicting. Faas *et al*, studying non-insulin dependent diabetic sujects (NIDDM), reported no difference in the fatty acid composition of diabetic erythrocytes or plasma, compared to controls (148), whereas Taylor *et al* reported higher linoleic acid and lower arachadonic acid content of erythrocyte membranes but, again, normal plasma fatty acid profiles in insulin dependent (IDDM) subjects (149). More recently, Pelikánová reported reduced levels of the essential fatty acids, linoleic and linolenic acid, and increased levels of their desaturated derivatives in the plasma and erythrocyte phospholipids, and plasma cholesterol esters, of NIDDM subjects (150). These differences could be explained in terms of the presumed action of insulin on FA metabolism, as insulin is believed to stimulate desaturase enzymes (150, 151) hence allowing, for example, the conversion of linoleic acid to arachadonic acid. Therefore it was proposed that in the relative insulin deficiency of IDDM such conversions would be reduced whereas, assuming that the insulin resistance of NIDDM applies less to FA metabolism than to glucose metabolism, in the hyperinsulaemia of NIDDM this mechanism would actually enhanced (150). If this were correct, NIDDM subjects would be more likely to have highly unsaturated, oxidisable, lipids in their lipoproteins then IDDM subjects. Although I have emphasised the similarities between NIDDM and IDDM with regard to atherosclerosis and so have argued that metabolic abnormaliteis in common to the two conditions are needed to explain their common association with atherosclerosis there are differences in the pattern of presentation of atherosclerosis in IDDM and NIDDM. Biochemical differences such as that proposed for PUFA metabolism would offer an explaination for the dissimilarities also in terms of FR mechanisms.

However, the whole idea that an increased PUFA:SFA in LDL might be disadvantageous seems at odds with epidemiological evidence that a rise in this ratio in the diet and tissues confers protection from vascular disease (152-154) and that diets high in saturated fats are associated with atherosclerosis. This anomaly may be explained by the fact that PUFAs cannot be regarded only as substrates for non-

enzymatic FR oxidation, nor can they be treated as one amorphous entity. There are many different PUFAs derived from the diet or synthesised from the two essential fatty acids, linoleic and linolenic acid (151). On the basis of structural similarities, and hence similar biochemistry, they fall into groups, linolenic acid and its derivatives comprise the Omega-3 group, linoleic acid, and derivatives, the Omega-6 group. Each PUFA has a unique function or set of functions, being more than passive structural components or sources of energy (155). They have roles in membrane structure and fluidity, potentially altering the function of cells (156). As substrates in enzymatic processes, they are converted to metabolically active substances such as thromboxanes and prostacyclins which help in the control of thrombosis, fibrinolysis and platelet function, processes also believed to be relevant in atherogenesis (157). Their influence on eicosanoid metabolism may also be involved in their ability to lower blood pressure (158). They have also been reported to influence plasminogen activator inhibitor and fibrinogen levels (159, 160). Lipoprotein metabolism is also influenced by PUFAs, especially those of the Omega-3 series (161) which consistently lower VLDL-triglyceride levels (162,163) although have less predictable effect on HDL and LDL levels (164,165). In non-diabetic subjects Omega-3 fatty acids also generally lower LDL concentration however in diabetics they may cause deleterious changes in LDL and glycaemic control (166-168).

Thus, whilst PUFAs are generally more oxidisable, and so theoretically more atherogenic, this effect may be completely confounded by the complicated balance of their specific effects on other factors important in the genesis of atheroma. However all of these effects may need to be considered if manipulations of PUFA:SFA ratio are to be attempted in efforts to reduce atherogenesis (169) and the need for caution is suggested by work such as that of Brown *et al* who showed that PUFA supplementation, in the form of fish oil concentrate (Maxepa), was associated with evidence of increased lipid peroxidation (TBARS) in normal subjects. Their work also suggested that unless accompanied by vitamin E supplementation, PUFA could actually cause increased whole blood aggregation and elevation of plasma glucose (170).

1.4. LOW DENSITY LIPOPROTEIN OXIDATION IN RELATION TO ATHEROSCLEROTIC RISK FACTORS OTHER THAN DIABETES.

I have argued that the biochemical abnormalities of diabetes are compatible with the proposal that it is a state in which more FR might be produced and more FR damage might occur. In turn this offers an explanation for the fact that diabetes is a risk factor for atherosclerosis which is consistent with the oxidation of LDL theory of atherogenesis. For the latter theory to hold it should also offer explanations for the association between atherosclerosis and the other risk factors. As so many risk factors, or more correctly, risk markers, are associated with atheromatous cardiovascular disease (ACVD), I will limit myself to a brief discussion of only the major ones.

1.4.1 Hypertension

Hypertension is one of the major risk factors for ACVD (171). Whereas 2-3 yrs of antihypertensive treatment reduced stroke mortality by a factor predicted from earlier epidemiological studies, the same is not true for coronary artery disease mortality (172,173). In fact, whilst reviewing evidence from 14 randomised trials of antihypertensive drugs Collins *et al* concluded that lowering blood pressure reduced coronary artery disease mortality, at best, by only half the epidemiologically expected amount (172). Although this observation may be explained by the fact that the drugs used to treat hypertension in trials to date cause deleterious effects in other risk factors, such as glucose tolerance and plasma lipids (81, 173), other explanations are also possible. One such possibility is that hypertension is not actually a major cause of ACVD but another manifestation of a more basic abnormality which causes both hypertension and ACVD. Treating hypertension would then not necessarily be expected to alter the course of ACVD.

Even in non-diabetic individuals, insulin resistance (IR) and hyperinsulinaemia have been noted to be strongly associated with hypertension and other ACVD risk factors (174). In addition plasma insulin concentration has been identified as an independent risk factor for the development of coronary artery disease (175) thus it has been proposed that IR and hyperinsulinaemia may be responsible for, and so provide the link between, both the development of hypertension and ACVD (174). The mechanisms involved are unclear but hyperinsulinaemia may act via effects on the

sympathetic nervous system (176), the renal handling of Na^+ (177), or transmembrane ion pumps, to modulate blood pressure (178). These same systems may also be involved in the control of cell growth and proliferation and so insulin may stimulate the smooth muscle cell proliferation seen in atheroma via these mechanisms however insulin may also act more directly to stimulate atheroma, via its own growth promoting activity (179). The apparent contradiction of proposing that insulin driven mechanisms may be responsible for hypertension despite the existence of insulin resistance hinges on the concept that insulin resistance predominantly affects insulin sensitive glucose utilization and that other insulin modulated activities remain insulin sensitive (174). Apart from suggesting a mechanism for the association of hypertension with atherosclerosis, if insulin resistance and hyperinsulinaemia are the basic defects explaining both this would also explain the association of hypertension and atherosclerosis with other insulin resistant states such as non-insulin dependant diabetes, obesity, impaired glucose intolerance and even with insulin dependant diabetes if it is accepted that peripheral hyperinsuliaemia is a feature of insulin treated insulin dependant diabetes (180). However, there are anomalies with this theory in that hyperinsulinaemia is not always associated with hypertension such as in the situation of insulinoma (181), other factors must be involved, and sol would like to speculate that FR activity might also be a useful area in which to look for a link between hypertension, atherosclerosis and diabetes.

I have already argued that FR processes may be important in the development of atheroma and may be increased in diabetes. There is also evidence that FR may be involved in blood pressure control. One of the most important mediators of resistance vessel relaxation is the endothelial derived relaxation factor (EDRF), which has been identified as nitric oxide (NO) (182, 183). NO is degraded by reaction with $O_2^{\cdot-}$ (183, 184) hence, in circumstances of increased FR load with either increased $O_2^{\cdot-}$ production, or reduced removal, impaired vascular relaxation might occur and lead to hypertension. Preservation of NO might then explain the reported acute antihypertensive effect of bolus injections of the antioxidants vitamin C, glutathione and thiopronine which was observed in diabetic and hypertensive human subjects (185). In keeping with a role for FR in disrupting endothelial relaxation is the work of Cameron *et al* (186). Endothelial derived relaxation of smooth muscle is believed to be reduced in diabetes (183) but in streptozotocin-diabetic rats Cameron *et al* found that this abnormality could be corrected by an

aldose reductase inhibitor (ARI) (186). A mechanism proposed to explain this effect relates to the fact that aldose reductase inhibitors preserve NADPH which is a co-factor for NO synthesis and, as discussed before, is also necessary for antioxidant recycling and protection against FRs such as $O_2^{\cdot-}$ (186). In addition, there is evidence that increased FR activity does occur in essential hypertension with the finding that lipid peroxides are elevated in the plasma and erythrocytes of hypertensive patients (187). Why FR activity might be increased in hypertension is unknown but could theoretically be due to its association with IR. Even when the IR associated with essential hypertension is of insufficient degree to cause glucose intolerance or frank diabetes with hyperglycaemia it is nevertheless associated with some subtle metabolic abnormalities which overlap those of diabetes (188). Essential hypertension was found to be accompanied by impaired insulin mediated glucose disposal and possibly insulin mediated inhibition of lipid oxidation (188). One could hypothesise that these metabolic abnormalities due to IR wihtout diabetes would have consequences in terms of FR production similar to those postulated for diabetes but of lesser degree. Thus a spectrum of biochemical alterations is envisaged with increasing IR being accompanied by increasingly abnormal metabolism with increasing FR production and accompanied by hyperglycaemia and manifest diabetes at its extreme. With the developement of diabetes, hyperglycaemia would then in turn add further to the FR load by the mechanisms described in 1.3.1.

1.4.2. Cigarette Smoking

Cigarette smoking is another of the major cardiovascular risk factors, many mechanisms have been proposed to explain this association. Smoking may have effects upon platelet aggregation, thrombolysis and fibrinolysis (189, 190). Also, atherogenic changes in serum lipid profiles are associated with smoking (191) but these changes alone are not believed to be enough to account for the increased risk incurred by smoking (191, 192). In fact, it has recently been postulated that these lipid changes themselves are secondary to the existence of insulin resistance and hyperinsulinaemia in smokers (193). As discussed, syndromes of insulin resistance are associated with cardiovascular disease and could possibly be associated with increased FR activity but there are more obvious associations between smoking and FRs.

Among the many toxic substances contained in cigarette smoke are a vast array of

FRs (13, 194, 195) which could cause direct injury to endothelial walls and also induce FR oxidation of LDL. Indeed, it has now been shown that smoking enhances oxidation of plasma lipoproteins, an effect which can be inhibited by antioxidant vitamins (196). Consistent with this, Duthie *et al* reported that levels of plasma diene conjugates were higher, and vitamin C concentrations were lower, in smokers (197). In addition, they also showed that, although erythrocyte vitamin E levels were not reduced in smokers, H_2O_2 induced peroxidation of erythrocytes was increased in smokers and could be corrected by vitamin E (197). By increasing FR load, smoking may cause a fall in antioxidant levels due to their increased consumption and low vitamin E and C levels have been found to correlate with risk of ischaemic heart disease (198, 199).

1.4.3. Gender

The differences in incidence, and timing, of coronary artery disease between men and women is largely unexplained (200) in terms of the major risk factors as is the loss of the protection conferred by female gender beyond the menopause and in diabetes (6) but FR mechanisms may be involved. Female sex may confer protection from cardiovascular disease by being associated with reduced availability of the transition metal, and FR catalyst, iron, due to losses in menstruation (201). Ferritin levels, and so iron stores, are known to be lower in pre-menopausal women than in men and it is particularly interesting that protection from vascular disease is lost even when the "menopause" is induced surgically by hysterectomy wihtout oopherectomy (202).

1.4.4. Age

Age may be associated with cardiovascular disease by simply being another process resulting from the accumulated effects of FR attack. One of the theories of aging is that it is itself a FR process (13, 203). At a biochemical level the changes in structural molecules that occur with aging, such as fluorescence and cross-linking of proteins, are similar to those induced by FR damage and to those that occur in diabetes (75). It is interesting, in the context of arguments regarding oxidative metabolism as a source of FRs, that one of the features of the condition of accelerated aging, progeria, is an increased metabolic rate (204) and that when

comparing different species, aging rate is generally correlated with metabolic rate (203). Also, in rats food restriction, which induces protective adaptive reductions in metabolic rate, results in delayed aging and prolongation of life (205). These observations are consistent with the idea that the more rapid the metabolism the more rapid the process of aging and this correlation results from the more rapid production of FR during metabolic activity.

Further suggestion that FRs are implicated in the damaging effects of more rapid metabolic rates is provided by the finding that when species are compared in terms of tissue concentrations of antioxidants relative to metabolic rate, then long lived species are found to have the highest relative antioxidant concentrations (203). Also, the activities of enzymatic antioxidand defences have been shown to alter with age (206) and processes associated with aging such as Alzheimer's disease, have been associated with reductions in vitamin E, A and carotenoid levels (207).

1.4.5. Geography And Social Class

Many possible explanations are proposed for the wide geographical and social class variation of atherosclerotic vascular disease. Dietary differences are one of the more frequently proposed explanations and diet may influence FR mechanisms by altering the balance between substrates for FR attack and antioxidants (152, 153, 198, 199). However, the atherogenicity of a diet will be determined by a complicated balance of the positive and negative effects of ingested PUFA, SFA, cholesterol and antioxidants on many processes which could influence atherosclerosis (169).

Whilst not proof, the ability of the oxidation of LDL theory to offer a model explaning the observed associations between risk factors and atherosclerosis does lend the theory indirect support. The major identified risk factors do have possible associations with FR mechanisms and so could operate through a common final pathway of FR oxidation of LDL to generate atherosclerosis. I have summarised in Fig 1.4 a proposed model of these interactions and I have indicated the position, as possible final common pathway, held by FR mechanisms in the generation of atherosclerosis.

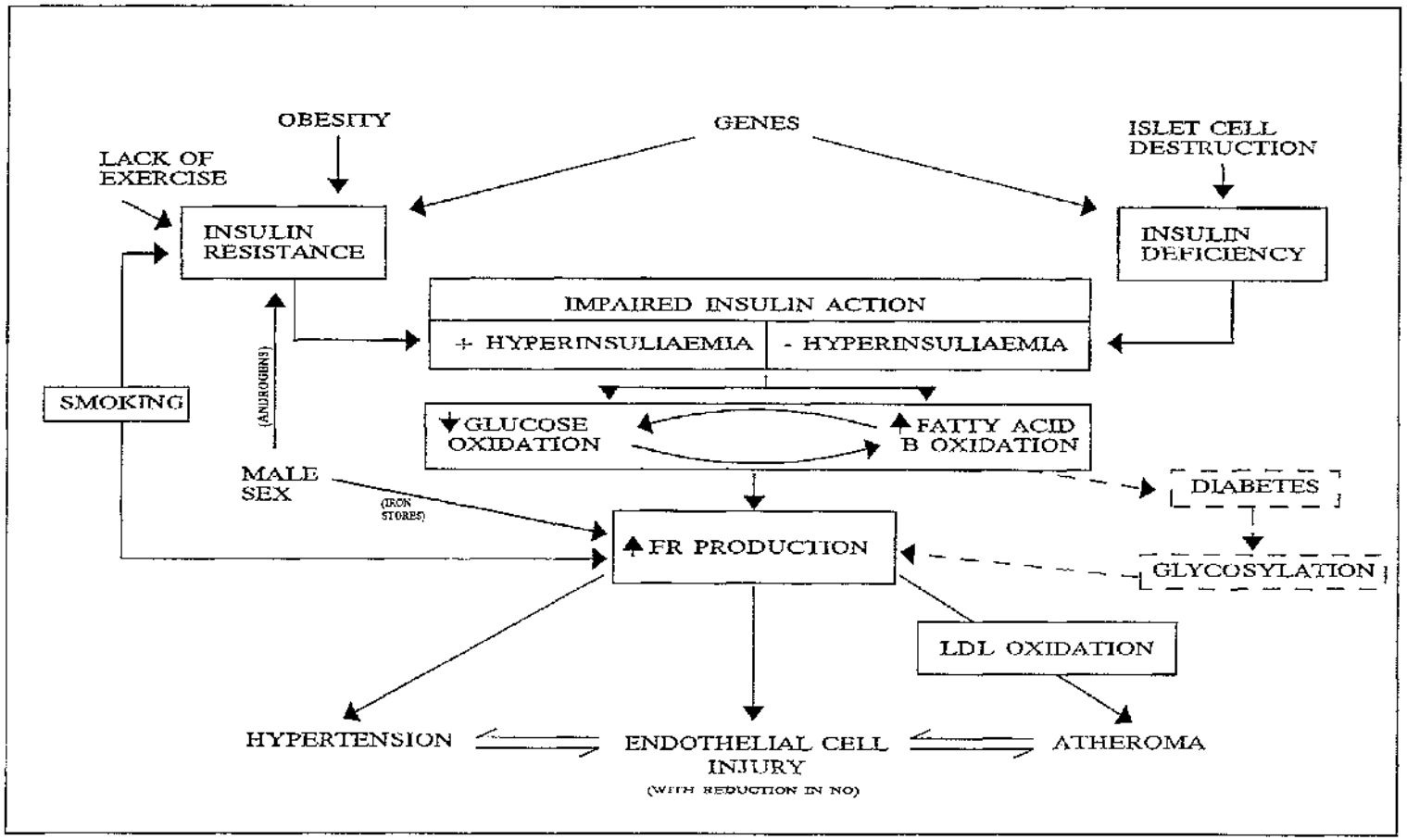


Figure 1.4: The interelationships between atherosclerotic risk factors, indicating the proposed role of free radicals.

1.5. CONCLUSIONS AND HYPOTHESES TO BE TESTED.

In summary, a compelling mechanism for the genesis of atheroma has been proposed involving the FR oxidation of LDL. The theory gains interest as a result of its ability to suggest explanations for, and new approaches to investigating, the associations between atheroma and its major risk factors. Should any of these currently speculative explanations be proven correct they would also lend power to the theory. This thesis investigates the links with diabetes.

Diabetes is a major risk factor for atherosclerosis, therefore, it seemed reasonable to ask might this association arise because more FR oxidation of LDL occurs in diabetes. There is some evidence from DCFA and TBARs concentrations that increased lipid oxidation does occur in diabetes but results are conflicting. Also, these markers of lipid oxidation are not specific and one of the unanswered methodological problems in this area was the role of diet in determining DCFA levels. The first study in this thesis was designed to test the hypotheses that :-

1. Diet is an important source of the diene conjugated fatty acids (DCFAs) in human serum and tissues, thereby questioning the use of DCFAs as a marker of FR activity.

One of the circumstances in which more FR oxidation of lipids might occur in diabetes would be if diabetes was a state in which more FR production and release occurred. Proposed and reported sources of increased FR production in diabetes have been discussed. Since platelet eicosanoid metabolism involves FR intermediates and is more active in diabetes, the second study explored the hypothesis that :-

2. Platelets are capable of releasing superoxide anion ($O_2^{\cdot-}$) and those from diabetic subjects release more than those from nod-diabetic subjects.

Impairment of the mechanisms protecting against FR attack would be a second situation in which more FR oxidation of lipids could occur in diabetes. There are reports of altered antioxidant concentrations in diabetes but, because co-operation between antioxidants occurs, it was not clear that measurement of the concentrations of selected species reflected overall antioxidant capacity. Using an assay reflecting

total antioxidant capacity, TRAP, I set out to determine if plasma from diabetic subjects did have reduced total antioxidant activity, testing the hypothesis that :-

3. Plasma total antioxidant activity, as measured by TRAP, is lower in diabetic subjects than in healthy controls, thereby exposing diabetics to increased risk of FR mediated damage.

and :-

4. The extent to which the lipids in plasma from diabetic subjects peroxidise will be inversely related to plasma total antioxidant activity, as measured by TRAP.

Changes in the composition of LDL in diabetes might also allow for increased FR oxidation of lipids. Vitamin E is not only a major lipid soluble antioxidant but is also an integral component of lipoprotein particles, including LDL. The vitamin E concentration in diabetic plasma has been reported to be normal but the concentration, per se, may not be the relevant parameter, it may be more important to determine vitamin E concentration relative to the concentration of lipids. In this thesis I have employed the ratio of vitamin E to cholesterol plus triglyceride concentrations to reflect vitamin E status and to clarify the situation as regards the appropriateness of vitamin E levels in diabetes. A study was undertaken to test the hypothesis that :-

5. Individuals suffering from diabetes have lower vitamin E levels relative to levels of oxidisable lipid (vitamin E status) than do non-diabetics and lower vitamin E status will be associated with evidence of greater lipid peroxidation in diabetes.

In keeping with the theory that oxidation of LDL by FR is important in the generation of atheroma is epidemiological evidence that the risk of atherosclerotic disease is greatest in populations ingesting diets low in antioxidants. A study was begun to test the hypothesis that :-

6. Vitamin C and vitamin E dietary supplementation will increase the TRAP and vitamin E status of plasma respectively, and reduce lipid peroxidation in

diabetics and non-diabetics.

CHAPTER TWO

METHODS

2.1. INTRODUCTION

Several standard methods are used in more than one of the studies in this thesis and are described here. A few methods which are specific to a single study, or employ non-standard procedures and need explanation in the light of the study, are described in the relevant chapters. Where an understanding of the principle of the assay is necessary for interpretation of the results, the principle is described in the relevant chapter(s). Where a standard kit assay is employed, and provides all the reagents, the reagents are not listed individually. Where coefficients of variability (CV) are given they refer to between batch estimates unless stated otherwise. Ethical approval was sought and obtained where necessary from the Whittington Hospital Ethical Commitee.

2.2 SAMPLE COLLECTION AND STORAGE

For all assays whole venous blood was collected into a plastic syringe from an antecubital vein using a 19G needle and tourniquet. Blood was immediately transferred to collection tubes containing appropriate anticoagulants or antioxidants as indicated. Serum was obtained by collecting blood without anticoagulants. and following coagulation and centrifugation at 1000g for 10min, serum was removed and stored as described.

2.3. ASSAYS

2.3.1. Plasma Glucose

Blood was collected into "Vacutainer" tubes containing sodium fluoride (Vacutain-system, Becton-Dickson, Cowley, Oxford, UK.) and centrifuged at 1000G for 10 min. Plasma was separated and immediately analyzed using the GLUCOSE OXIDASE METHOD (Beckman, Brea, CA). (CV = 2.0%)

2.3.2. Glycosylated Haemoglobin (HbA_{1c})

Blood was collected into "Vacutainer" tubes (Vacutain System. Becton-Dickinson, Cowley, Oxford, UK.) containing ethylenediamidetetracetic acid (EDTA) and stored at 4°C. All samples were analyzed within 3 days of collection by AGAR GEL ELECTROPHORESIS (Ciba-Corning, Halstead, Essex, UK). (CV = 4.3%)

2.3.3. Serum Lipids

Serum was stored at 4°C and analyzed within 4 days using standard kits.

a. Total Serum Triglycerides (TG)

Following lipase hydrolysis, the total concentration of TG was estimated by an ENZYMIC COLORIMETRIC TEST with glycerol phosphate oxidase and 4-aminophenazone (Roche, Welwyn Garden City, Herts., UK), run on a Baker CentrifiChem 600 (Baker Inst.,Liverpool, UK). (CV = 3.4%)

b. Total Serum Cholesterol (total-Chol)

Following cholesterol esterase hydrolysis, the concentration of t-Chol was estimated by an ENZYMIC COLORIMETRIC METHOD using cholesterol oxidase and 4-aminophenazone (Boehringer-Mannheim, Lewes, Sussex, UK.), run on a Baker CentrifiChem 600. (CV = 1.7%)

c. High Density Lipoprotein Cholesterol (HDL-Chol)

Following phosphotungstic acid (0.55mmol.l^{-1}) precipitation of VLDL, LDL and chylomicrons, leaving only HDL in the supernatant (Randox Lab Ltd., Crumlin, Antrim, UK) the remaining cholesterol in the supernatant was determined by the above method. (CV = 4.6%)

d. Low Density Lipoprotein Cholesterol (LDL-Chol)

LDL-Chol concentration was determined by calculation using the Friedewald equation (208). Since the assumptions made in deriving this equation do not hold when hypertriglyceridaemia exists, LDL-Chol was not calculated when the TG concentration exceeded 5.0mmol.l^{-1}.

2.3.4. Serum Uric Acid

Uric acid was determined by an ENZYMATIC COLORIMETRIC ASSAY employing Uricase (Randox Lab Ltd., Crumlin, Antrim, UK), run on a Baker CentrifiChem 600. (CV = 2.8%)

2.3.5. Serum Vitamin E (vitamin E)

Serum was analyzed immediately or stored at -20°C. Samples were analyzed within 2 months by the method described by Hansen and Warwick (209).

Methanol/hexane extraction was followed by measurement of the fluorescence of the hexane layer in a Perkin-Elmer MPF-36 spectrofluorimeter (Perkin-Elmer, Beaconsfield, Bucks., UK) with excitation wavelength of 295nm and emission wavelength at 330nm. 0.2ml 20μgml^{-1} of Vitamin E, in HPLC grade methanol, was used as a standard and 0.2ml HPLC grade water as the blank. Vitamin E concentration of the serum is then calculated from

$$\text{Vitamin E } \mu\text{gml}^{-1} = \frac{F_t}{F_s} \times 20$$

Where :-

F_t = Fluorescence of test corrected for the blank

F_s = Fluorescence of standard corrected for the blank

(CV = 4.8%)

Taking the molecular mass of vitamin E as 430.7 the vitamin E concentration was converted to umol.l^{-1} by multiplying by a factor of 2.3.

2.3.6. Total Peroxyl Radical Trapping Activity Of Plasma (TRAP)

The principle behind this assay and the derivation of the equation for calculation of TRAP is given in Chapter 5. TRAP was estimated using a modification of the method described by Wayner *et al* (127). Serum was stored -20°C and used within one month.

3.0ml of phosphate buffered saline was placed in the chamber of a Clark O_2 electrode (Rank Brothers, Bottisham, Cambridge, UK), stirred continuously and allowed to equilibrate to 37°C. 2μl of linoleic acid (free acid, 99%) was dispersed into the buffer, giving a final concentration of 2.1mmol.l^{-1}, followed by 50μl of serum. Finally, 30μl of 0.4 mol.l^{-1} 2,2'-azo-bis(2amidinopropane) hydrochloride (ABAP) was added. The chamber was then covered to exclude light. The output from the electrode was recorded by means of a Vitatron-2000 recorder (MSE/Fisons

Inst., Crawley, UK). After a variable time lapse (T_p), due to plasma inhibition of peroxidation, O_2 uptake accelerates as peroxyl radicals derived from the decomposition of ABAP attack and peroxidise lipid in the reaction mixture (first peroxidation phase). When the rate of O_2 uptake had become constant, 25μl of 500μmol.l^{-1} ascorbic acid was added. Peroxidation was inhibited for a second period (T_a). When peroxidation resumed and reached a steady state, recording was stopped.

T_p and T_a are determined by measurements from the recording and are proportional to the antioxidant activity of plasma and the added ascorbate respectively (Fig 5.3 and 5.4). TRAP is calculated as follows:-

$$\text{Since } \frac{T_p}{T_a} = \frac{\text{[TRAP] x Vol. plasma}}{\text{[Conc. Ascorbate] x n x Vol. ascorbate}}$$

Where n = the number of peroxyl radicals trapped per ascorbic acid molecule and was taken as 1.7 (26).

$$TRAP = 425 \times \frac{T_p}{T_a}$$

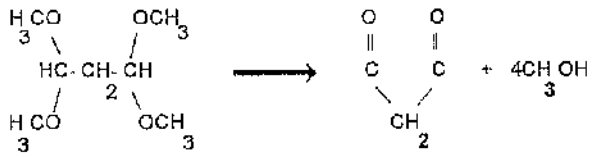
Where samples were analyzed in duplicate the results varied by less than 10%.

2.3.7. Serum Thiobarbituric Reactive Substances (TBARS)

The principle behind this assay is described in Chapter 6. TBARS were estimated using a modified fluorometric method of Yagi (210). Serum was stored at -20°C and used within 2 months

a. The Standard (≡ MDA 0.25μmol.l^{-1})

Since malondialdehyde (MDA) is not stable, it is formed during the course of the test from 1,1,3,3-tetramethoxypropane which hydrolyses under the test conditions, releasing MDA.



1,1,3,3- Tetramethoxypropane → Malondialdehyde + Methanol (MDA)

Stock Standard Solution (0.25mmol.l^{-1})

1.62ml of 1,1,3,3-tertamethoxypropane was diluted to 40ml with methanol. This was stored at 4°C in the dark.

Working Standard Solution (0.25μmol.l^{-1})

The stock solution was diluted by 10^6 in HPLC grade water immediately before use.

b.TBA Reagent (23.3mmol.l^{-1})

0.335g TBA was dissolved in 50ml water and heated to 80°C. After cooling, 50ml glacial acetic acid was added. The solution was stored in the dark at 4°C.

c.TBARS Assay

Acidification and precipitation of lipoproteins

To 50μl of serum 4ml 0.042mol.l^{-1} sulphuric acid and 0.5ml 0.035mol.l^{-1} tungstophosphoric acid were added and allowed to stand for 5min. After centrifugation at 1000G for 10min, the supernatant was discarded and the tubes drained by inversion. The sediment was resuspended in 4ml water.

Reaction with TBA

1ml TBA reagent was added to each sample and to 2ml of working standard. A blank was prepared by adding 1ml TBA reagent to 4ml water. Samples, standard

and blank were heated in a waterbath for 60min at 95°C and then cooled.

Extraction of the chromogen

3.0ml of n-butanol was added to all tubes and the contents mixed vigorously for 3min. After centrifugation at 1000G for 15min, the upper butanol layer was removed.

Measurement and calculation of TBARS

A Perkin-Elmer MPF-3L scanning spectrofluorimeter (Perkin-Elmer, Beaconsfield, Bucks., UK) was used, with an excitation wavelength of 515nm and emission wavelength of 553nm. Correcting for the fluorescence of the blank, the fluorescence of the test sample (F_t) and of the standard (F_s) were determined and TBA reactivity, expressed in terms of MDA, was calculated as follows:-

Since :-

$$\frac{[MDA]_t \times Vol_t\ (ml)}{[MDA]_s \times Vol_s\ (ml)} = \frac{F_t}{F_s}$$

$$[MDA]_t \mu mol\,l^{-1} = \frac{F_t}{F_s} \times [MDA]_s \times \frac{Vol_s}{Vol_t}$$

Where :-

$[MDA]_t$	=	conc. of MDA in test serum ($\mu mol.l^{-1}$)
$[MDA]_s$	=	conc. of MDA in standard ($\mu mol.l^{-1}$)
	=	$0.25 \mu mol.l^{-1}$
Vol_t	=	volume serum (ml) = 0.05ml
Vol_s	=	volume standard (ml) = 2ml
F_t	=	peak fluorescence of serum, corrected for the blank
F_s	=	peak fluorescence of standard, corrected for the blank

Then :-

$$MDA_t = 10 \times \frac{F_t}{F_s} \mu mol\,l^{-1}$$

(CV = 12.0%)

2.3.8 Total Diene Conjugated Fatty Acid (tDC) In Serum

tDC was determined using a modification of the method of Lunec *et al* (20). Lipids are extracted from serum and diene conjugated species detected by their ability to absorb at 230-240nm. Serum was stored at -20°C and used within 2 months.

a. Extraction

4ml of chloroform:methanol (2:1 v:v) was added to 0.5ml of sample and vortexed for 3min. After centrifugation at 1000G for 10min, 2.5ml of the lower phase was separated and vortexed with 1ml HPLC grade water for 30sec. Following a further centrifugation, the lower chloroform layer was removed to a quartz cuvette with a 1cm light-path length.

b. Measurement

Absorbance at 240nm was measured using a Pye-Unicam 8-100 recording spectrophotometer (Pye-Unicam, Cambridge, UK). Results, in OD units, represent tDC.

2.3.9 Serum Phospholipid Content Of Linoleic Acid (18:2(9,12)) And It's Diene Conjugated (18:2(9,11)) Derivative

The serum phospholipid content of 18:2(9,11) and 18:2(9,12) isomers were determined by a modification of the method of Ivensen *et al* (211). Serum was stored at -80°C and used within 2 months.

a. Hydrolysis Of Phospholipids

100μl of serum was mixed with 100μl of a solution containing 0.1mol.l^{-1} tris (hydroxymethyl)aminomethane (TRIS), pH 8.9, 1mol.l^{-1} methanol and 5000U.l^{-1} phospholipase A_2. This mixture was incubated at 25°C for 15min.

b. Addition Of Internal Standard

50μl of methanol, containing 0.5% acetic acid and 10μmol.l^{-1} of 18:2(9,11) linoleic acid was added.

c. Extraction Of Fatty Acid And Precipitation Of Proteins

300μl of 0.042M sulphuric acid and 1.5ml of chloroform:methanol (2:1 v:v) were added and the mixture vortexed. After centrifugation at 1000G for 10min three layers form; an upper methanol layer, a lower chloroform layer and an intermediate protein layer. The chloroform layer was removed and evaporated to dryness under O_2-free nitrogen. The residue was resuspended in 100μl of the HPLC mobile phase.

d. Fatty Acid Separation, Detection And Measurement

20μl of the resuspended sample was applied to a Spherisorb OD52, 250 x 4mm, HPLC column, containing 5μm capped spherical particles (Hichrom, Reading, Berks., UK). The mobile phase was acenotnitrile/water/acetic acid (90:10:0.1). Two U.V. detectors in series were set at 234nm and 200nm to detect diene conjugated and non-conjugate fatty acid, respectively. Fatty acid concentrations were calculated using a programme devised by Griffin (212).

e. Percentage Molar Ratio (%MR)

The %MR of 18:2(9,11) to 18:2(9,12) concentrations was calculated as follows:-

$$MR\% = \frac{[\text{conc } 18{:}2(9,11)]\ \mu mol.l^{-1}}{[\text{conc } 18{:}2(9,12)]\ \mu mol.l^{-1}} \times 100$$

2.3.10. Peroxidisability Of Serum Fatty Acids

The principle behind this assay is discussed further in Chapter 6.

1 volume of serum ($\simeq$ 2ml) was mixed with 2 volumes of 0.2mol.l^{-1} sodium acetate buffer to acheive a final pH of 5.5. Immediately samples were removed for estimation of tDC and TBARS by the methods described in Sections 2.3.8 and 2.3.7 respectively. The mixture was then incubated at 37°C in the dark for 24hrs. At the end of that period, samples were removed for tDC and TBARS estimates and the change in these concentrations from baseline were calculated.

2.3.11. Low Density Lipoprotein (LDL) Isolation

LDL, in the density range d1.020-d1.050g.ml^{-1}, was isolated by potassium bromide (KBr) density gradient ultracentrifugation. 20ml of blood was collected into glass tubes, on wet ice, containing sodium ethylenediamine tetracetic acid (EDTA) and Butylated Hydroxytoluene (BHT) to achieve final concentrations of 1g.l^{-1} EDTA and 4.4mg.l^{-1} BHT. Tubes were centrifuged at 1000G for 10min at 4°C and the plasma removed. Further manipulations were carried out with plasma on wet ice.

Solid potassium bromide (KBr) was added to 5ml plasma in centrifugation tubes (Beckman, UK) to adjust density to 1.020g.ml^{-1}. The tubes were capped and mixed until the salt was dissolved.

Using a 50.3Ti rotor, installed in a Beckman L8-55 ultracentrifuge, samples were spun for 18hr at 39000 rpm at 4°C. The supernatant (VLDL/IDL) was removed by tube slicing. The infranatant (LDL/HDL/plasma proteins) was made up to 5ml using d1.020gml^{-1} KBr salt solution (28.5g KBr in 1l deionised, distilled water). The density of the sample was then increased to 1.050 by further addition of solid KBr (0.218g/5ml). The tube was then capped, mixed and ultracentrifuged as before, but for 24hr. Supernatant LDL was removed by tube slicing and its volume adjusted to 2.0ml with d1.05 salt solution. This stock solution of LDL was sealed and stored at 4°C, in the dark for no more than one week. It was dialysed immediately before use.

2.3.12. Dialysis Of Low Density Lipoprotein

Dialysis was performed as described by Esterbauer *et al* (213) against 100-fold volume of degassed 0.01M phosphate buffered, 0.16M saline, pH 7.4 containing 0.1μg.ml^{-1} chloramphenicol. Dialysis was performed over 48hrs in the dark at 4°C.

2.3.13. Low Density Lipoprotein Protein Content

Protein content was determined COLORIMETRICALLY (Sigma, Poole, Dorset, UK) employing the Lowry method (214).

2.3.14. Oxidation Of Isolated Low Density Lipoprotein

The principle behind this assay is discussed further, and the equations derived, in chapter eight. The method employed is a modification of the method described by Esterbauer (213). The protein content of dialysed LDL was first determined by the Lowry method then dialysed LDL was diluted to achieve a final concentration of 0.25mg.ml^{-1} (in terms of LDL protein) in O_2-saturated, 0.01M phosphate buffered, 0.16M Sodium Chloride, pH 7.4. Fresh copper sulphate (final concentration 1.66μM) was added to catalyse oxidation and the mixture was maintained at 37°C. The change in absorbence at 234nm was monitored using a Pye-Unicam SPB-100

Spectrophotometer (Pye-Unicam, Cambridge, UK) and has three phases as shown in Fig 8.1. The duration of the lag phase, (LAG), the duration of the propagation phases, (PROP), and the maximal change in absorbence were measured (ΔAb). Taking the extinction co-efficient (ϵ_{234}) of conjugated lipid peroxides to be 29500$M^{-1}cm^{-1}$, and bearing in mind the 0.25$mg.ml^{-1}$ concentration of LDL, the nanomoles of DC formed per mg of LDL (DC-mg^{-1}) and their rate of formation (DC-RATE) were calculated as follows :-

$$DC\text{-}mg^{-1} = \frac{\Delta Ab}{\epsilon_{234}} \times 10^6 \times 4 \text{ nmoles } mg^{-1} \text{ LDL}$$

$$DC\text{-}RATE = \frac{\Delta Ab}{\epsilon_{234}} \times \frac{10^6 \times 0.25}{PROP}$$

Where LAG was estimated on duplicate samples results varied by 5-10%

2.4. SOURCES OF REAGENTS

BDH Chemicals Ltd, Poole, Dorset, UK, supplied Analar grade:-

acetic acid (glacial), ascorbic acid, n-butanol, betahydrotoluene (BHT), copper sulphate, methanol, sodium acetate, sodium chloride, sulphuric acid, tungstophosphoric acid, potassium *di*-hydrogen orthophosphate, and *di*-sodium hydrogen orthophosphate.

They also supplied "Spectrasol" grade chloroform.

Sigma Chemical company Ltd., Poole, Dorset, UK, supplied:-

ethylenediamine-tetra-acetic acid (EDTA), linoleic acid, malondialdehydetetramethylacetal(1,1,3,3-tetramethoxypropane),phospholipase A_2, thiobarbituric acid (TBA), α-tocopherol (vitamin E) and tris(hydroxymethyl)aminomethane (TRIS).

Rathburn Chemical Ltd., Walkerburn, Peebleshire, UK, supplied:

HPLC grade water, acetic acid and acetonitrile.

2,2'-azo-bis-(amidinopropane hydrochloride) (ABAP) was supplied by Polyscience Incorporated, Northampton, UK.

2.5. SUBJECTS: DEFINITION OF CLINICAL CHARACTERISTICS

The groups of subjects who took part in the different studies are described in the relevant chapters.The presence or absence of the complications of diabetes was established by patient history, supplemented by reference to the patient's notes. Subjects were classified as having macrovascular complications as follows:-

(a) Ischaemic heart disease:	previous myocardial infarction or history of angina or an abnormal ECG.
(b) Peripheral vascular disease:	claudication or two more absent foot pulses.
(c) Cerebrovascular disease:	previous cerebrovascular accident or history of transient ischaemic event

Subjects were classified as having microvascular complications as follows:-

(a) Diabetic retinopathy:	abnormal dilated fundoscopy recorded by experienced fundoscopist.
(b) Diabetic nephropathy:	albustix positive proteinuria recorded on the day of study and on one or more previous visits (urinary tract infection excluded).
(c) Neuropathy:	symptoms or signs of sensory impairment or absent ankle jerks recorded.

2.6 STATISTICAL METHODS

Statistical analyses were performed using SPSS (SPSS-INC., Chicago, USA.) or C-Stat (Chartwell Scientific Publishing Limited, Oxford, UK.) statistical packages.

For Normally Distributed data results are given as mean (standard deviation). Comparison between groups was tested by paired or unpaired Student t-tests and correlations between continuous variables evaluated by linear regression analysis. Pearson correlation co-efficients (r) and their level of significance (p value) are quoted for the results of linear regression analysis.

For non-normally distributed data results are expressed as median (25^{th}, 75^{th} percentiles). Comparison between groups was tested by Wilcoxon Signed Rank Test (paired data) or Mann-Whitney U-test (unpaired data). Correlation between variables was evaluated by Spearman rank test. Spearman rank correlation coefficients (r_s) and their p values are quoted.

For Categorical data the distribution within groups was compared by the Chi-squared test. Chi-squared statistic (χ^2) and p value are given.

Possible interactions between variables were evaluated by multiple linear regression analysis (MLR) or analysis of covariance, with logarithmic transformation of skewed data. Categorical variables were coded as follows:- male (1), female (0); smoker (1), non-smoker (0); presence of diabetes (1) and its absence (0). Results of MLR are described by partial regression coefficients (B) with their 95% confidence intervals (95%CI) and p values. For analysis of variance results are described by the variance ratio (F) and p value. Statistical significance was accepted at a p value of 0.05. Estimates of statistical power were made using the calculations and tables described by Cohen (215).

CHAPTER THREE

DIET AS A SOURCE OF PHOSPHOLIPID ESTERIFIED 9,11-OCTADECADIENOIC ACID IN HUMANS

3.1 INTRODUCTION

Free radicals are believed to be mediators of tissue damage in many disease states (11,15,18, 21), including diabetes (70, 123, 216) and atherosclerosis (9,217). However, much of the evidence for such proposals is based on indirect measures of FR activity because direct techniques, such as electron spin resonance, are not widely available. The most frequently used indirect method of measuring FR activity is the measurement of the products of FR attack on endogenous molecules, especially lipids. I will describe the reactions involved in the process of FR attack on lipids and how the major products are generated as this knowledge is necessary to aid the understand of the measurements made.

3.1.1. Free Radical Attack On Lipids And Peroxidation (Fig 3.1)

PUFA are vulnerable to FR attack because of the arrangement of double bonds in their side chains. The usual configuration of double bonds found within PUFA side chains in biological material is termed unconjugated meaning that the double bonds are separated by at least two single bonds (-C=C-C-C=C). This arrangement provides a "weak spot", as far as FR attack is concerned, at the methylene groups ($-CH_2-$) which interrupt the double bonds (13, 15, 218) because FR attack and hydrogen atom abstraction ($H^{\cdot}$) from this group (Fig 3.1) is relatively favoured by the fact that the single electron can then be delocalised along the unsaturated chain. *Hydrogen abstraction* leaves a carbon centred radical. An electron rearrangement follows resulting in the loss of the unconjugated configuration and the production of a diene conjugated system, where double bonds are separated by only one single bond (-C=C-C=C-) (218). The process continues with the reaction of the carbon centred radical with O_2, giving rise to a highly reactive peroxyl radical. The peroxyl radical is then capable of *propagating* the chain reaction by abstracting a $H^{\cdot}$ from another suitable PUFA residue whilst itself becoming a *diene conjugated*

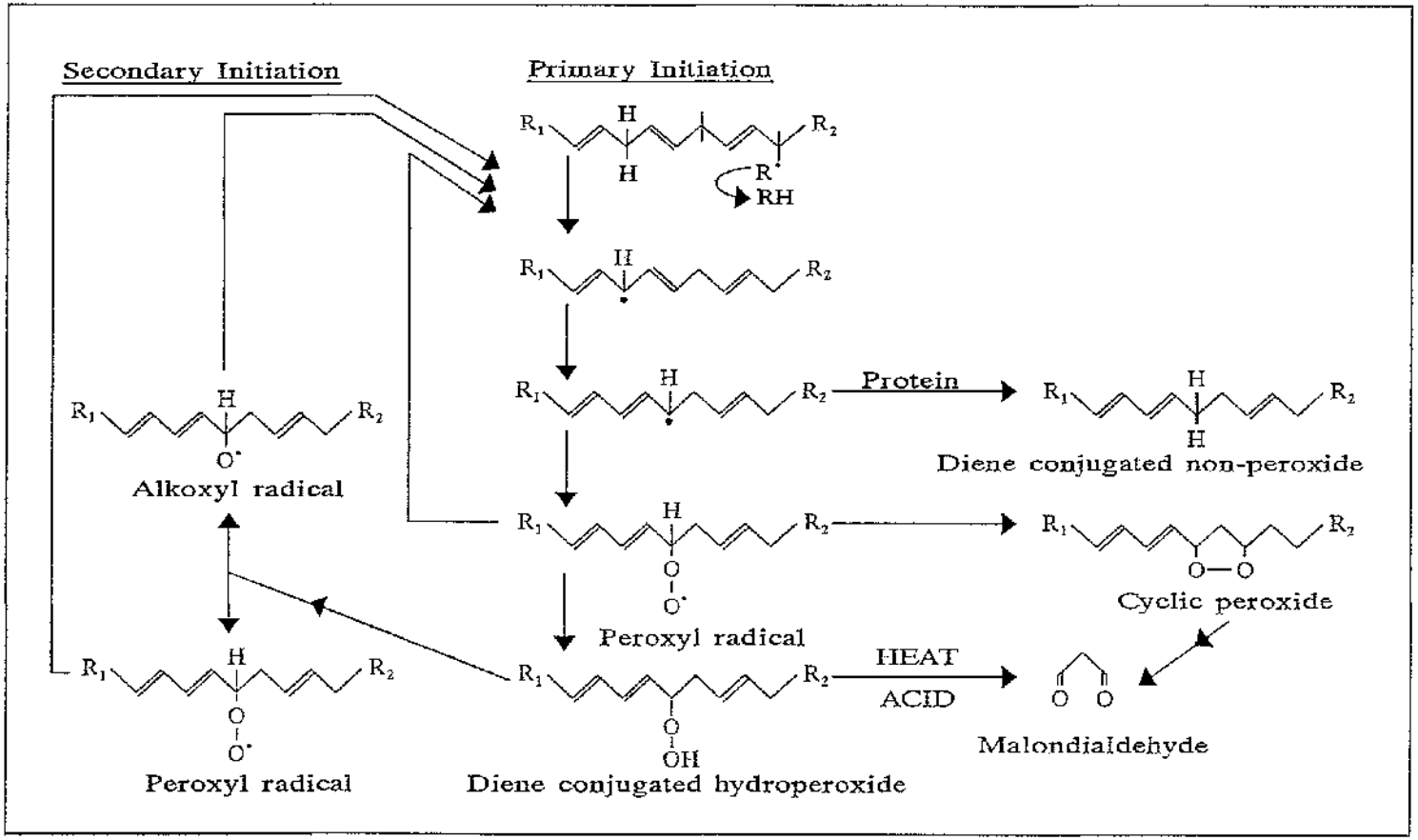


Figure 3.1: Free radical attack on a polyunsaturated fatty acid.

hydroperoxide (15, 219). This peroxidation sequence is believed to be the major reaction pathway followed when a FR attacks a PUFA hence the term peroxidation is often used as if synonymous with FR attack on lipids but this is not strictly correct as another pathway may also exist. In the presence of protein, reaction with O_2 need not occur and FR attack can then result in isomerisation and the formation of *diene conjugated, non-peroxide products* (Fig 3.1) (218, 220).

These initial FR products ,especially the hydroperoxides, may undergo further reactions and fragmentation, with the formation of a series of compounds, the exact nature of which is determined by the original PUFA. These compounds include aldehydes such as malondialdehyde (MDA), and gases such as pentane and methane. Also, hydroperoxides may react in the presence of transition metals to produce other radicals such as alkoxyl and peroxyl radicals (Fig 1.2) which may then initiate secondary chain reactions (Fig 3.1) (13, 221).

All of the species formed in the above reactions have been found in biological samples, suggesting such processes do occur *in vivo* and many of them have been used as markers of FR activity *in vivo*. DCFA are one of the most stable and most frequently measured products however recently other potential sources of DCFA have been identified raising doubts about the specificity of DCFAs as FR markers. These other sources are discussed below and the study described in this chapter explores the possibility that diet is a source in humans.

3.1.2. Alternative Sources Of Diene Conjugated Species

Using HPLC with U.V. detection it has been shown that the major DCFA in human tissues is 9,11-octadecadienoic acid which is the 18:2(9,11) isomer of linoleic acid (9,12-octadecadienoic acid) (220). Assuming its only source to be FR attack on the parent isomer the percentage molar ratio (%MR) of 18:2(9,11) to 18:2(9,12) in biological samplescould then be used as an index of FR activity (211, 220). However, the same entity has been found in animal tissues where it arises as a result of enzymatic processes, as in rat liver microsomes where desaturation of 11-octadecaenoic acid (18:1(11)) to 18:2(9,11) occurs (222). Also, the bacteria residing in the bovine rumen can dihydrogenate 18:2(9,12) to octadecanoic acid (18:0) via an 18:2(9,11) intermediate (223). Similar processes may occur in human tissue, for example, local production and raised concentrations of DCFAs in the human cervix may result from bacterial colonization and activity (224). Also, several respiratory

pathogens have been shown to produce 18:2(9,11) *in vitro* (225).

In addition to the possibility of non-FR production of 18:2(9,11) in human tissues, the fact that this isomer appears in animal tissue suggests that ingestion may be another source for humans (72). Food-stuffs, mainly those of ruminant origin, have been shown to be rich in 18:2(9,11) (226). Fogerty *et al* have shown food-stuffs to have a very wide range of 18:2(9,11) content for example, lean beef contained 6-43mg of 18:2(9,11)/100g (227). This fatty acid was mainly distributed in the fat component of the meat, beef fat containing 960-1310mg/100g in addition butter was found to contain 720-910mg per 100g. Fogerty *et al* also showed that some foods of non-ruminant origin, such as margarine, contained no 18:2(9,11), but that some contained a range of concentrations explicable in terms of the animal food chain. Eggs contained up to 31mg/100g but eggs from hens feeding free-range with no animal fat source having no detectable 18:2(9,11) (227).

Thus, controversy existed as to the origin of DCFA in human tissues and to the importance of diet as a source. The study described in this chapter was designed to clarify the situation by observing the effect of altering the intake of foodstuffs high in 18:2(9,11) upon the serum phospholipid content of 18:2(9,11).

3.2 HYPOTHESIS

Diet is an important source of diene conjugated fatty acids (DCFAs) in human serum and tissues, thereby questioning the use of DCFAs as a marker of FR activity.

3.3 SUBJECTS AND STUDY DESIGN

Fourteen healthy subjects (3 males, 11 females; mean age 35.1 (SD 8.7) yrs took part in the study. All were non-smokers.

Two diets were devised using the work of Fogerty *et al* (227) to identify foodstuffs rich in 18:2(9,11). The "high diet" was rich in these foodstuffs, whereas the "low diet" was poor in such foodstuffs. Stringent dietary instructions were not given, but volunteers were simply given a list of foodstuffs (Table 3.1) either to avoid or to increase their intake of. Subjects recorded a 7-day diet history on each phase of the diet. These were scored allowing a score of 1 for a typical portion of each item appearing on the prescribed list of foodstuffs. Because previous work has shown an enormous range of 18:2(9,11) concentration in food of similar origin (227), we did

1	Red meat:beef, lamb, pork, ham.
2	Meat products:pies, sausages, pasties, meat spread
3	Animal fats:butter, lard.
4	Dairy foods:dairy ice-cream, cream soups and sauces full fat cheeses, milk, yoghurts.

Table 3.1: Foodstuffs rich in the 18:2(9,11) isomer of linoleic acid.

Subjects were asked to increase the intake of these foodstuffs during the 'high diet' phase and to reduce their intake during the 'low diet' phase.

not attempted to use any more than semi-quantitative score of its intake. Semi-skimmed milk was scored as 1 and full fat milk as 2 (per half pint). An average daily score of number of items eaten was calculated. All subjects were studied at baseline on their normal diet and again after a period of 3 weeks on one of the test diets. Where both diets were undertaken, the order was randomized and a washout period of at least 3 weeks was allowed between phases. Four subjects undertook both dietary phases, four only the "high diet" and six only the "low diet".

3.4 METHODS

The analytical and statistical methods employed are described in chapter 2. All assays were performed within 8 weeks of sample collection. Serum samples taken before and after each dietary phase were analysed in the same batch. Serum phospholipids were subjected to enzymatic hydrolysis and DCFAs were determined by HPLC with u.v. detection at 234nm. Concentrations of 18:2(9,11) and 18:2(9,12) were determined and expressed as a percentage molar ratio (%MR):-

$$\%MR = \frac{\text{18:2(9,11) LA x 100\%}}{\text{18:1(9,12) LA}}$$

3.5 RESULTS

The daily diet score of subjects on the"low diet" was significantly lower than at baseline on their normal diet [0.6(0.5) vs 2.6(0.7) items/day, n = 10, p = 0.0003]. On the "high diet" it was significantly higher [5.3(0.8) vs 2.9(1.0) items/day, n = 8, p = 0.001].

The concentration of 18:2(9,11) increased from 12.1(3.7) to 18.8(7.4) μmol.l^{-1} on the "high diet" (p = 0.006) and decreased from 14.3(6.7) to 8.9(4.7) μmol.l^{-1} on the "low diet" (p = 0.01). Correcting for changes in 18:2(9,12) by expressing the value as the %MR to 18:2(9,12), the %MR increased on the "high diet" [1.3(0.4) vs 1.9(0.7); p = 0.01] and decreased on the "low diet" [1.6(0.6) vs 1.1(0.4); p=0.004] (Fig 3.2). Of the 18 pairs of results, 17 changed in the direction predicted by the hypothesis. Eleven subjects kept diet histories, allowing 25 pairs

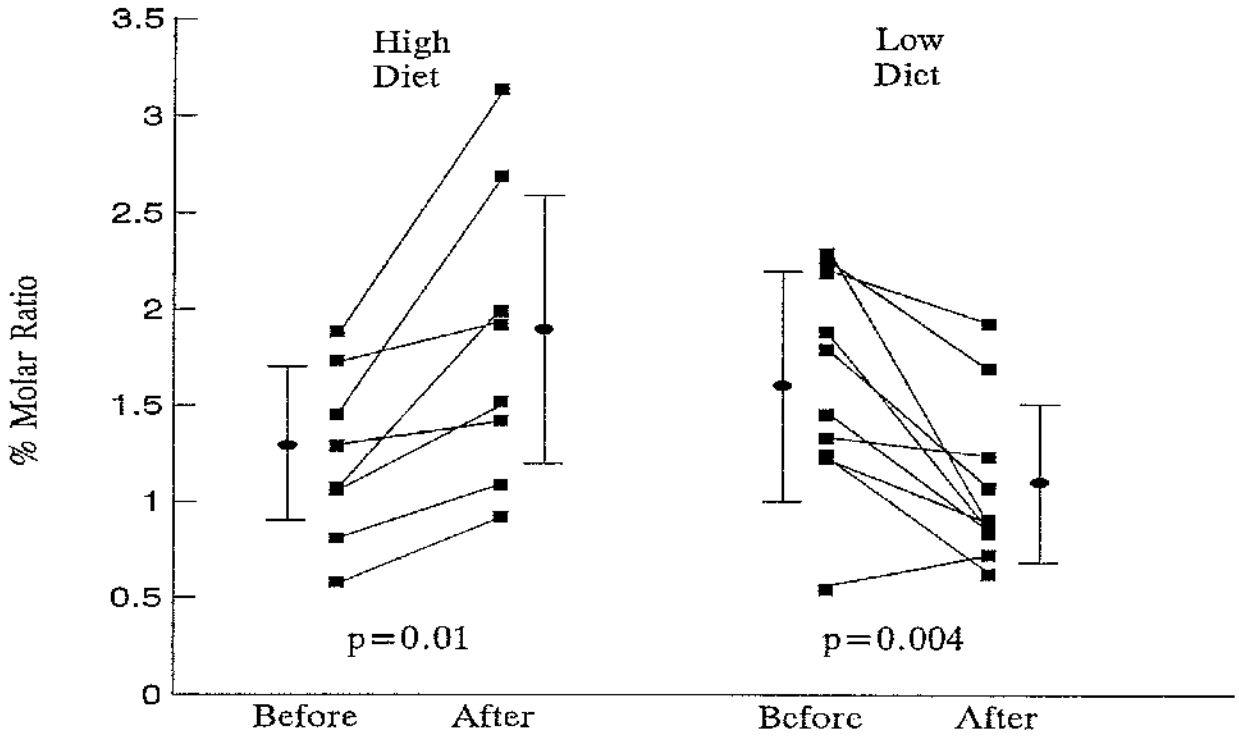


Figure 3.2: The effect of "High" diene conjugate and "Low" diene conjugate diet on %molar ratio of 18:2(9,11) to 18:2(9,12) linoleic acid.

of diet scores and related %MR be analysed. There was no correlation between baseline diet score and baseline %MR. When all diet scores and %MR were analysed together, there was highly significant correlation between diet score and %MR ($r = 0.636$, $p = 0.001$)(Fig 3.3). The change in diet score and the change in %MR also correlated significantly ($r = 0.829$, $p = 0.001$) (Fig 3.4).

3.6 DISCUSSION

The aim of this study was to determine whether changes in the dietary intake of 18:2(9,11) would be reflected in the serum concentration of that entity thus suggesting that diet contributed to the variability in it's serum concentration. Since I wished the test diets to be different for that individual, but remain realistic, dietary instructions were given to subjects as simple guidelines, rather than the more artificial recommendation of strictly measured quantities of defined foodstuffs. It was felt this less rigid approach would also improve compliance.

The results support the hypothesis that the serum concentration of 18:2(9,11) is influenced by dietary intake. Despite deliberately, uncontrived test diets, the percentage change in %MR in human serum on the "high diet" ranged from 10% to 88% and on the "low diet" from 7% to 60%. This suggests that normal dietary variation in a population would also be sufficient to have a major influence on serum concentration of 18:2(9,11).

Seventeen of the eighteen dietary interventions produced changes in the %MR in the direction predicted by the hypothesis. The one paradoxical result arose in the subject with the lowest baseline %MR (0.54). It may be that it is impossible to lower the concentration beyond a certain level, a level determined by factors other than diet.

Dietary compliance and 18:2(9,11) intake were estimated using a simple diet score. Acknowledging the inaccuracy of this method it was adopted for the following reasons. Unlike better recognised dietary components the 18:2(9,11) content of very few foodstuffs is known, therefore it does not appear in food tables. In known sources its concentration varies widely and will be greatly influenced by fat content (227). Therefore, even if careful weighed records of dietary intake had been kept, calculation of 18:2(9,11) content with any degree of accuracy would have been impossible. On the other hand, considering the items in Table 3.1, although a typical portion of red meat would weigh more than a typical portion of paté or

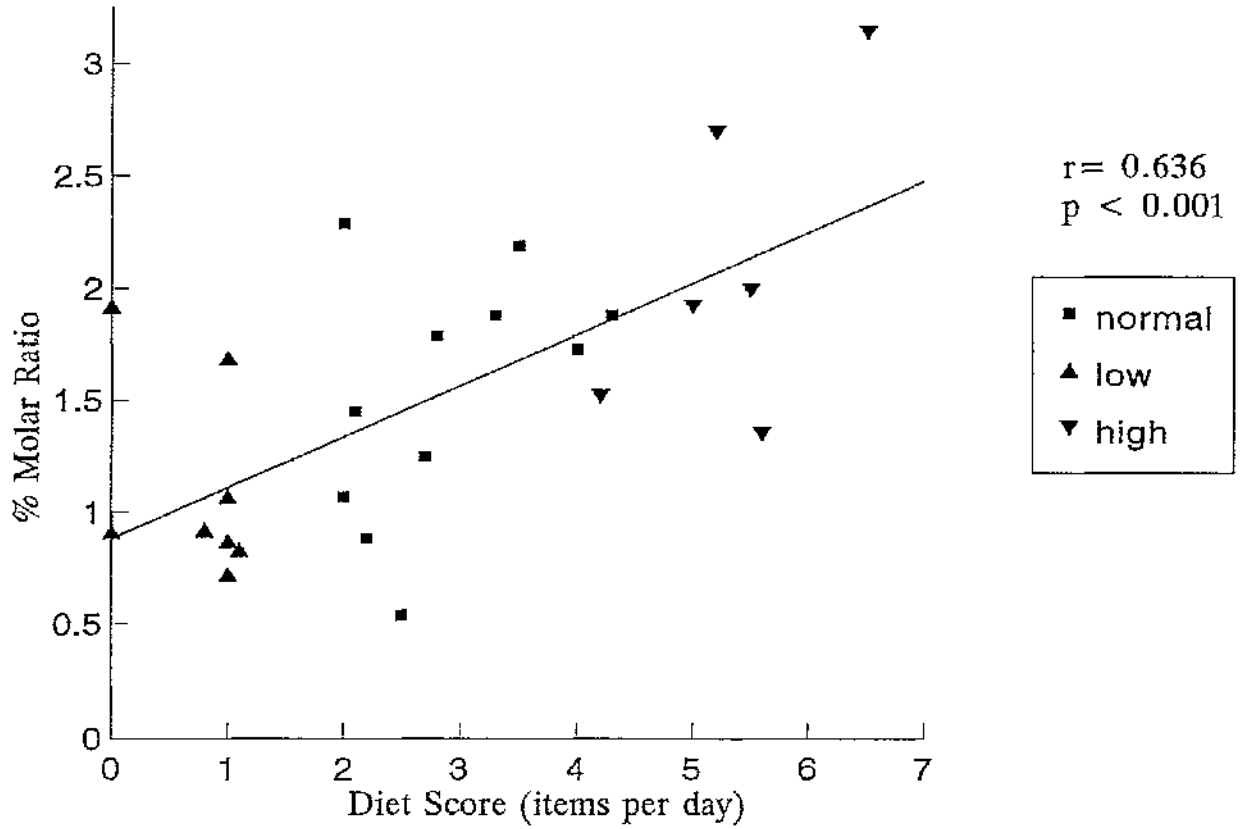


Figure 3.3: The relationship between diet score and %molar ratio of 18:2(9,11) to 18:2(9,12) linoleic acid.

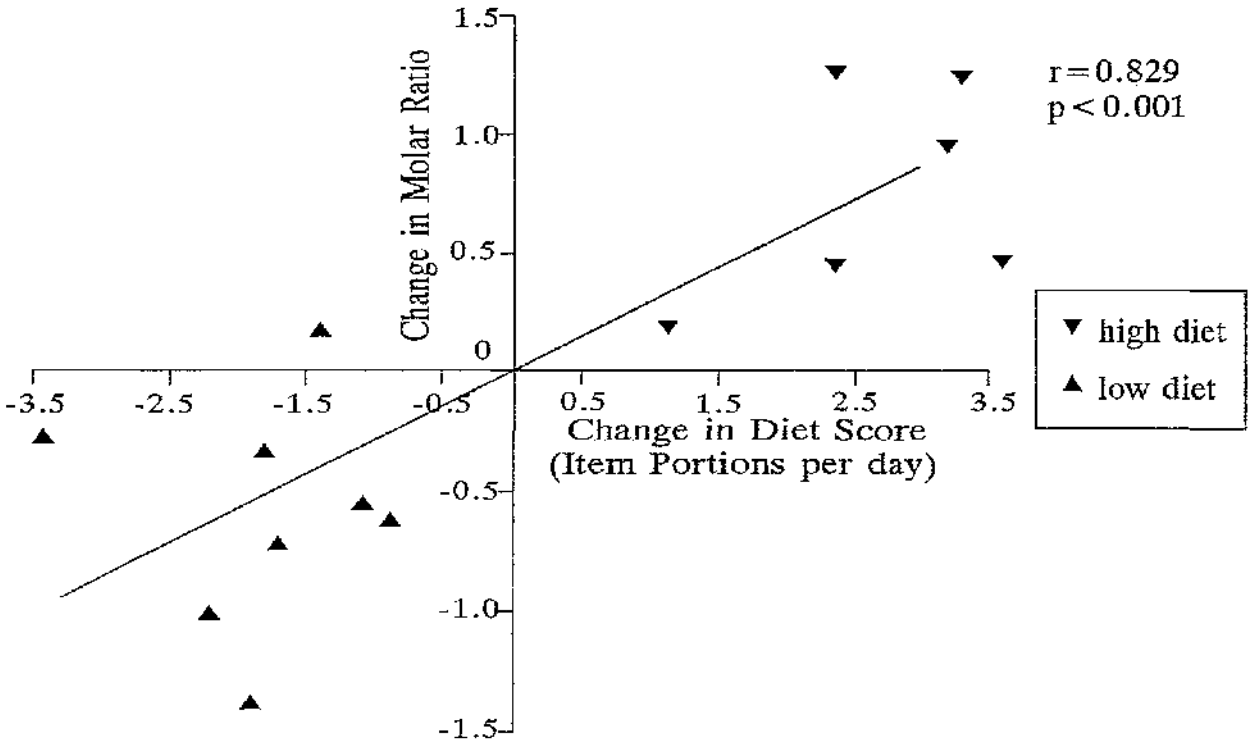


Figure 3.4: The relationship between the change in diet score and the change in %molar ratio of 18:2(9,11) to 18:2(9,12)linoleic acid.

cheese, its fat content would be less, therefore typical portions of these items might be assumed to be more equivalent in terms of 18:2(9,11) content than initial inspection would suggest. Also, the errors introduced by this assumption of near equivalence are likely to be small relative to the differences produced in the comparison between presence or absence of a typical portion of these items in the diet for example, changing butter (720mg 18:2(9,11)/100gm) for margarine (0mg/100gm) will produce such relatively large alterations in 18:2(9,11) intake as to swamp errors in equivalence between portions of steak and paté. Moreover, any inaccuracy of this method would be likely to disguise, rather than exaggerate, any relationship with plasma DCFAs ratios.

The lack of correlation between diet score and %MR at baseline may be due to the inaccuracies of the diet score, may be a statistical problem owing to small numbers, or again may imply that other factors contribute to %MR besides diet. Free radical activity may be that other factor, but the extent of the influence of diet alone is so great as to confound the situation completely. When all pairs of dietary scores and %MR are considered there is a significant correlation (Fig 3.3). The fact that the regression line for diet score vs %MR does not pass through zero also suggests a minimum concentration produced by sources other than those identified here. There is also remarkably good correlation between change in diet score and change in %MR (Fig 3.4), suggesting that the dietary assessment and scoring system does identify the major contributors to serum concentration despite its simplicity. Some workers have found low levels of DCFA in a group of young insulin-dependent diabetic patients, despite the theory that in diabetic patients FR activity is increased and relates to complications (71). It is possible that these findings could be explained by the subjects consuming diets low in animal fats. Similar considerations may explain my findings of lower levels of DCFA in a group of Asian diabetic patients than in a Caucasian group, despite the former being more at risk of cardiovascular complications (228).

3.7 CONCLUSION

In conclusion, the use of DCFA as a marker of free radical activity when comparing groups or individuals over a prolonged period is limited, as diet is perhaps the major source of these compounds in humans. The magnitude of the day-to-day variation in DCFA levels within an individual has not been studied here. If this were not

great, sudden major alterations might still be attributed to bursts of FR activity in short-term longitudinal studies, for example within hours of a significant event such as myocardial infarction (229) or major surgery (230).

CHAPTER FOUR

PLATELETS AS A SOURCE OF SUPEROXIDE.

4.1. INTRODUCTION

The explanation for the greater cardiovascular risk associated with diabetes is unclear. As discussed in chapter one a compelling theory for the development of atheroma is evolving which suggests that oxidation of low density lipoprotein (LDL) renders it more atherogenic and evidence suggests that this oxidation is a free radical process (43-48) and so in situations where greater production of free radicals occured one might expect more LDL oxidation and greater risk of atherosclerosis. Platelets are more active in diabetes (113, 231) and have been attributed a role in the development of atheroma (232, 233) but the mechanism(s) whereby they might do so remain unclear. Histologically platelets are found in close association with macrophages in atheromatous plaques (234). They have been reported capable of enhancing macrophage accumulation of cholesterol esters (235) and, like macrophages, of enhancing oxidation of LDL (236). Platelets are not known to have an enzyme system specifically geared to the production of oxygen centred free radicals (eg superoxide, $O_2^{\bullet-}$) as macrophages do. However like stimulated macrophages, platelets stimulated to aggregate do display a burst of oxygen uptake attributed to mitochondrial electron transport and cyclooxygenase/lipoxygenase activation (237). Activity of the latter enzyme cascade is an integral part of the platelet response to many of the agonists of platelet aggregation and essentially it involves the insertion of molecular oxygen into arachidonic acid with the production of prostaglandins, thromboxanes and leukotrienes (13, 238). *In vitro* purified prostaglandin synthetase and lipoxygenase have been shown capable of $O_2^{\bullet-}$ production (16).

In this chapter the possibility is explored that a mechanism explaining the role of platelets in the generation of atherosclerosis is that they act as a source of FRs allowing the leak of oxygen centred free radicals such as $O_2^{\bullet-}$ during aggregation thereby contributing to oxidative stress and so to LDL oxidation. Free radicals may also cause direct injury to endothelial cells. These effects might be more marked in diabetic subjects since their platelets have been found to be more aggregable (113, 231). Particularly relevant to the current proposal is the finding of reduced vitamin

E concentration in diabetic platelets (125). Vitamin E is a free radical scavenger and a major lipid antioxidant, it also has a modulating effect on platelet function inhibiting platelet aggregation (109, 239). The aim of the study described in this chapter was to establish whether or not platelets did release $O_2^{\bullet-}$, especially during aggregation, and if so to assess whether the effect differed in extent in platelets from diabetic and non-diabetic subjects.

Like other radicals $O_2^{\bullet-}$ can act as either an oxidising or reducing agent. It's ability to reduce ferricytochrome C (Fc) to ferrocytochrome C (Equation 4.1), producing a change in absorbance at 550nm was made use of to enable it's detection.

Equation 4.1 $\quad Fc - Fe^{3+} + O_2^{\cdot-} \rightarrow Fc - Fe^{2+} + O_2$

Reduction of Fc is not specific to $O_2^{\bullet-}$ so that specificity of detection was ensured by looking only at that component of reduction inhibited by superoxide dismutase (SOD) (19, 24, 240).

Preliminary experiments were performed with platelets suspended in plasma but, as will be seen, plasma contained substances capable of Fc reduction and so to avoid this interference definative experiments were performed with platelets isolated from plasma by gel filtration.

4.2. HYPOTHESIS.

Platelets are capable of releasing $O_2^{\cdot-}$ and those from diabetic subjects release more than those from non-diabetic subjects.

4.3. STUDY DESIGN.

Diabetic platelets were harvested from diabetic subjects chosen at random from among routine attenders at the diabetic clinic. Non-diabetic platelets were harvested from normal healthy laboratory workers. Subjects were all fasting at the time of blood sampling. In preliminary experiments platelets in plasma were studied. In the definitive study platelets isolated from plasma by gel filtration were studied. All experiments were performed on multiple platelet samples, the exact number varying from experiment to experiment but as indicated in the results section.

4.4. METHODS.

Because the analytical and experiment methods used in this chapter are not used elsewhere they are described here as opposed to in the methods chapter.

4.4.1. Platelet Preparations.

a. Platelet Rich Plasma (PRP).

Whole blood was collected by venepuncture using a 19 gauge needle and immediately added to 3.8% citrate [9:1 vol:vol] followed by centrifugation at 150 G for 10 mins. The supernatant PRP was removed.

b.Platelet Poor Plasma (PPP)

PRP was centrifudged at 1,500 G for 10 mins. The supernatant PPP was removed.

c.Platelet Rich Suspension (PRS)

PRP was applied to a gel filtration column (diameter 2.5cm, height 8cm.) packed with cross linked sepharose 2B (Pharmacia, Milton Keynes, UK). The column was equilibrated by prior washing with the elution buffer (NaCl : 8 g/l; KCl : 0.2 g/l; NaH_2PO_4 : 0.065 g/l; $MgCl_2$: 0.415 g/l; $NaHCO_3$: 1 g/l, corrected to pH 7.3). All salts were Analar grade, (BDH, Poole, Dorset, UK). Those fractions judged visually to be rich in platelets were pooled to form PRS. The platelet rich suspension contained platelets at a mean count of 213 (SD 94) x $10^3.mm^{-3}$.

d. Platelet Poor Suspension (PPS)

PRS was centrifuged at 10,000 G for 1 min in a Beckman minifuge (Beckman Instruments, Inc., Brea CA, USA). The platelet free supernatant was harvested.

4.4.2. Platelet Aggregation

Platelet aggregation was achieved by agitating platelets with collagen (1 mg/ml in isotonic glucose) (Hormon-chemie, München, Germany) using such volumes as would give a final collagen concentration of 3 μg/ml in reaction mixtures. In the preliminary experiments agitation was achieved by stirring, whilst in the definitive experiments platelets were shaken.

The extent of platelet aggretion was determined by counting platelets using an Ultraflow 100 (Beckton-Dickinson. Abingdon, Oxon, UK).
In experiments in which platelet aggretion was assessed the fall in platelet concentration at 10 and 60 mins from that at 0 min was expressed as a percentage of that at 0 mins.

4.4.3. Ferricytochrome C Reduction And Superoxide Generation

Ferricytochrome C (Fc) type III and bovine erythrocyte superoxide dismutase (SOD) were obtained from Sigma (Sigma Chem Co, St Louis, Missouri, USA).

a. Preliminary Experiments- Fc reduction by stirred platelets in plasma

Fc, at a final concentration of 50μM, was stirred for 15mins with aggregating platelets (PRP+collagen), resting platelets (PRP-collagen) or with plasma (PPP+collagen). As a blank Fc was also stirred in the presence of collagen alone. The OD at 15 mins was measured after centrifigation at 10,000 G to remove platelets.

b. Definitive Experiments-Fc reduction and Superoxide Generation by gel filtered platelets.

The experimental procedure is depicted in Fig 4.1. SOD and Fc solutions were made fresh daily. The final concentration of Fc in the reaction mixture was increased to 200μM. Initial experiments were performed with larger volumes to allow sampling at more frequent intervals. Ultimately sampling was performed only at 10 and 60 min of incubation. Samples were immediately centrifuged (10,000 g for 1 min) to remove platelets, diluted and read at 550 nm in an LKB Biochem Ultrospec 4050 (LKB Pharmacia, Milton Keynes, UK).

4.4.4 Presentation Of Results

The effect "attributable to resting platelets" on Fc reduction is calculated as the difference between the OD at 550 nm after exposure to non-aggregating platelets (PRP or PRS) and that after exposure to a suspension free of platelets (PPP or PPS). In the definative experiments, and with reference to Fig 4.1, this means that the effect attributable to resting platelets was calculated as the difference between the

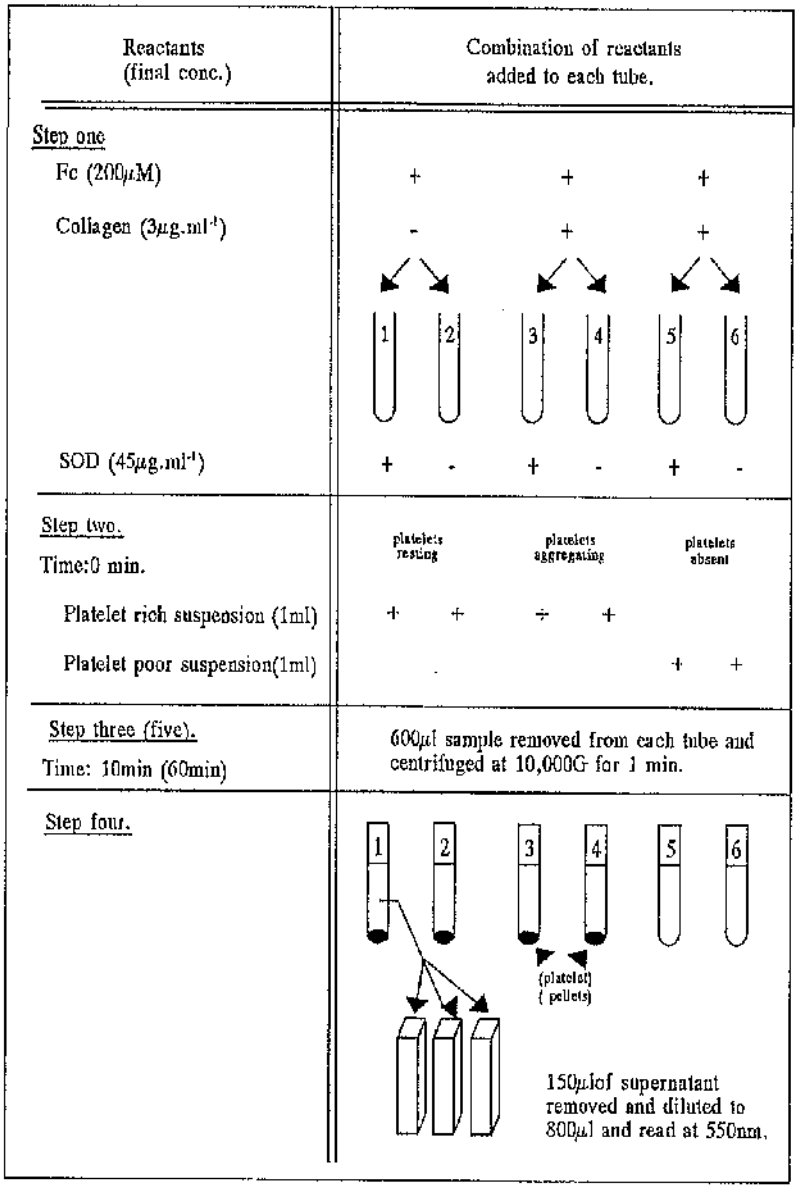


Figure 4.1: The experimental procedure used to investigate the reduction of ferricytochrome (Fc) by platelets in the presence and absence of superoxide dismutase (SOD).

OD found in samples from tubes 2 and 6. The difference between tubes 1 and 5 was also attributable to resting platelets but in the presence of SOD. Comparison of these differences in the presence and absence of SOD was used to determine the effect of SOD. The effect "attributable to aggregation" was calculated as the difference between OD after exposure to aggregating platelets (PRP or PRS agitated with collagen) with OD after exposure to non-aggregating platelets (PRP or PRS agitated without collagen) that is as the difference between tubes 3 and 5, with SOD, and between tubes 4 and 6, without SOD (Fig 4.1). Differences in OD are expressed in OD units and are corrected for platelet concentration by taking 100 x 10^3.mm^{-3} as unity. Results are shown as mean $\pm$ 1 standard deviation (SD) and are compared by paired and unpaired t-test.

4.5. RESULTS

4.5.1 Preliminary Experiments.

Using stirred platelets in plasma the OD of Fc rose significantly on exposure to both aggregating platelets [0.968 (0.079) vs 0.393 (0.017) OD units $p < 0.001$] and resting platelets [0.955 (0.112) vs 0.393 (0.017) OD units $p < 0.001$]. Therefore the effect attributable to aggregation, determined as the difference between the effects of exposure to aggregating vs non-aggregating platelets (resting), was small and not statistically significant (Mean difference 0.014 (0.041) $p=0.3$) (Table 4.1). However stirring with platelet poor plasma and collagen ,or with collagen alone, also produced a background reduction of Fc (Table 4.1). This background effect added a source of error and potentially masked platelet effects but could be eliminated by separation of platelets from plasma by gel filtration and shaking as opposed to stirring. Hence gel filtered platelets were used in further experiments.

4.5.2 Definitive Experiments.

The fact that gel filtered platelets were viable and that shaking supported aggregation was confirmed by the presence of visible aggregates in all experiments and by counting platelets to determine the drop in platelet numbers in representative experiments. Collagen induced aggregation of gel filtered, shaken platelets was significantly greater than that which occured spontaneously (Table 4.2). Also

	a	b	a-b	c	a-c	d
Fc without exposure (OD units)	Aggregating platelets (OD units)	Resting platelets (OD units)	Difference attributable to aggregation (OD units)	PPP + collagen (OD units)	Difference attributable to platelets (OD units)	Collagen (OD units)
0.393 (0.017) (n=10)	0.968 (0.079) (n=9)	0.955 (0.112) (n=9)	0.014 (0.041) p=0.3	1.006 (0.133) (n=8)	-0.050 (0.094) p=0.180	0.754 (0.151) (n=8)

Table 4.1: The reduction of Ferricytochrome C (Fc) by stirred platelets in plasma.

Results are the optical densities at 550nm following 15minutes exposure to aggregating platelets (PRP + collagen) or resting platelets (PRP - collagen). The effect of controls containing plasma + collagen and collagen alone are also included.
In "a" PRP was mixed with collagen, in "b" collagen was replaced by buffer.
Values are mean (SD) comparisons were tested by paired t-tests.

10 MINUTES SHAKING						60 MINUTES SHAKING					
Collagen induced			No collagen (spontaneous)			Collagen induced			No collagen (spontaneous)		
With SOD %	Without SOD %	Effect of SOD p	With SOD %	Without SOD %	Effect of SOD p	With SOD %	Without SOD %	Effect of SOD p	With SOD %	Without SOD %	Effect of SOD p
79.7(10.7) *p<0.001 n=11	72.0(22.3) *p=0.005 n=11	0.150	10.6(11.1) n=8	11.1(12.9) n=9	0.869	65.2(26.6) *p=0.02 n=8	57.0(24.4) *p=0.02 n=8	0.116	27.5(16.3) n=9	30.4(13.7) n=9	0.834

Table 4.2: The effect of superoxide dismutase (SOD) on platelet aggregation during shaking of gel filtered platelets with and without collagen.

Platelet numbers were counted before and after the period of shaking and the percentage aggregation calculated. Values are mean (SD) and comparisons between groups were tested using unpaired t-tests.

*p relates to the comparison between experiments in the presence and absence of collagen ie. between collagen induced and spontaneous aggregation.

spontaneous and collagen induced aggretation was not inhibited by superoxide dismutase (SOD) (Table 4.2).

The time course of Fc reduction on exposure to gel filtered, aggregating platelets is seen for three representative experiments in Fig 4.2. In subsequent experiments measurement of OD was performed only at 10 and 60 minutes.

Using shaken, gel filtered platelets (Fig 4.1) the absorbance of Fc exposed to resting platelets for 60 mins without SOD (tube 2) was not significantly different from that exposed to a platelet free preparation (PPS) (tube 6) in other words there was no significant effect attributable to resting platelets [1.128 (0.372) vs 1.107 (0.379) OD units, mean difference = 0.021 (0.062), p= 0.242] (Fig 4.3). Similarly insignificant differences were seen if the diabetic and non-diabetic subjects were analysed separately (Fig 4.3). The effect on Fc reduction attributable to aggregation was not signigicantly different in the presence or absence of SOD at 10 mins [WITH SOD: 0.009 (0.040) vs WITHOUT SOD: -0.026 (0.076) OD units, p=0.052] (Table 4.3) or at 60 mins [WITH SOD: 0.013 (0.064) vs WITHOUT SOD: 0.012 (0.101) OD units, p=0.987] (Table 4.3). There was no significant difference between diabetics and non-diabetics (Table 4.3).

4.6 DISCUSSION

No evidence of Fc reduction or superoxide anion release attributable to resting or collagen stimulated aggregating platelets was found in this study. Hence this study lends no support to the hypothesis that platelets release $O_2^{\cdot-}$ suggesting that this cannot be proposed as a mechanism whereby platelets contribute to atherosclerosis. In preliminary experiments it was found that plasma contained unidentified species capable of reducing Fc. The SOD inhibitable component of this reduction was not investigated here, but superoxide generation in serum has been reported (106) and glycated proteins and glucose autooxidation were suggested as possible sources (18, 102, 105, 106). The reduction of Fc seen in the presence of collagen alone cannot be explained. Because of anticipated problems in identifying potentially only small changes in absorption at 550 nm against this background methods of eliminating this interference were sought. This elimination was achieved when gel filtered platelets where used and shaken as opposed to stirred. The use of gel filtered platelets might explain differences in my results from those of Marcus *et al* (241). Using washed platelets they saw continuous SOD inhibitable reduction of Fc, and presumably $O_2^{\cdot-}$

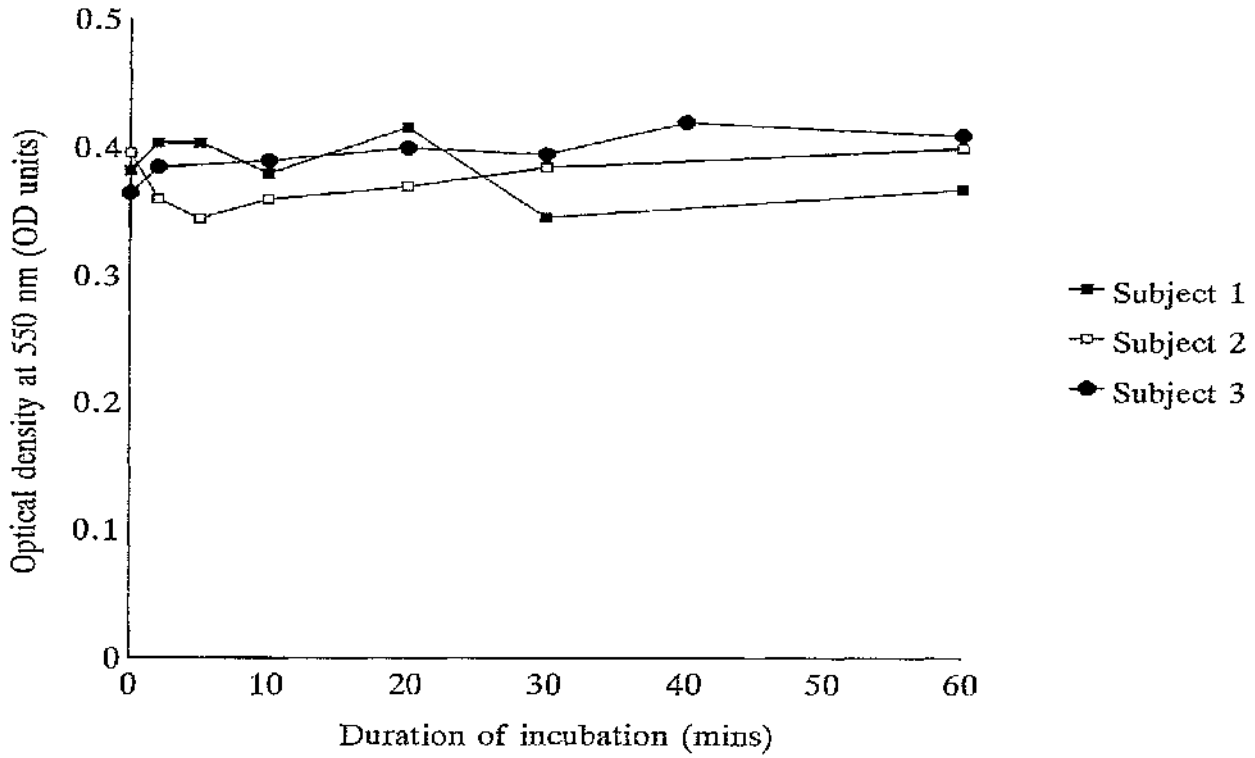


Figure 4.2: The time course of ferricytochrome C reduction in the presence of gel filtered, aggregating platelets.

Three typical experiments. Agonist was added at time zero. The extent of ferricytochrome C reduction is represented by the optical density of samples following centrifugation to remove platelets.

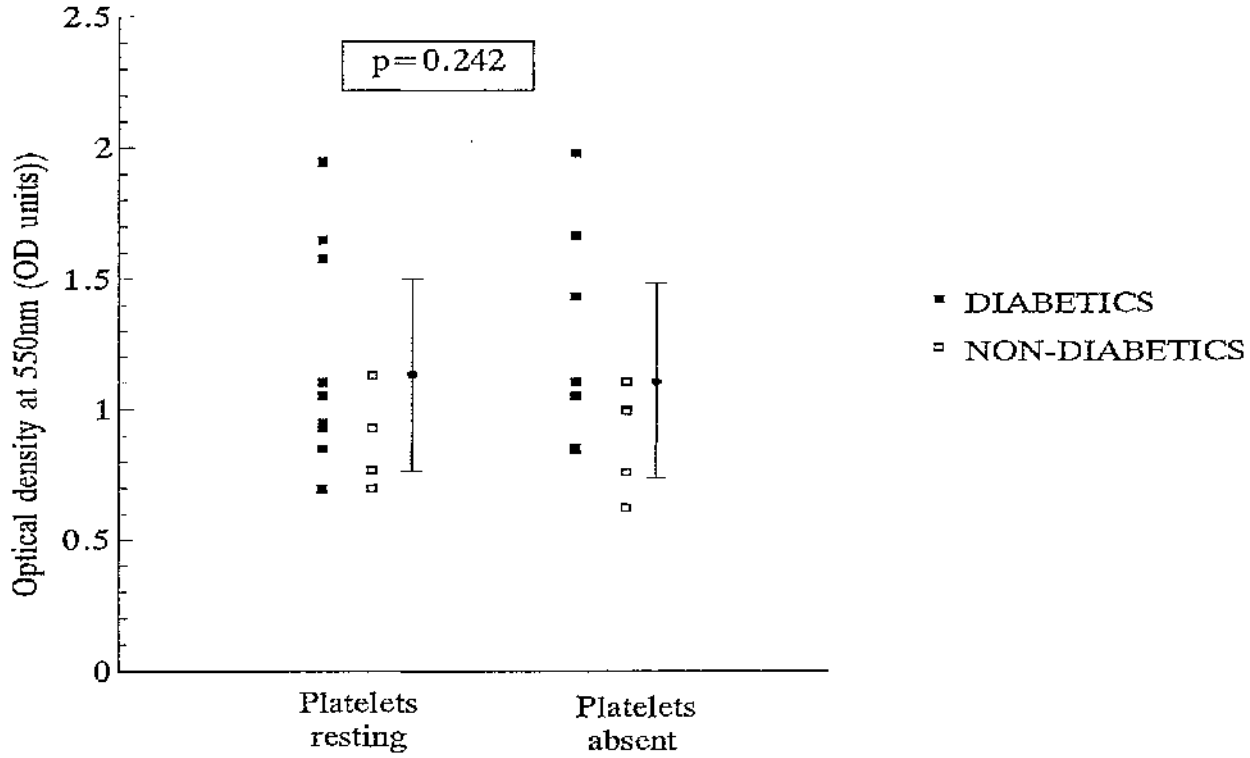


Figure 4.3: The effect of resting platelets on ferricytochrome C absorbance

Ferricytochrome C was incubated with resting platelets or in the absence of platelets. The results show the OD of ferricytochrome C after 60mins of such incubation in diabetic and nondiabetic subjects. Samples were centrifudged to remove platelets before absorbance measurements.

	ABSORBANCE ATTRIBUTABLE TO AGGREGATION (OD UNITS)					
	10 MINUTES			60 MINUTES		
Subjects	With SOD (tube3-5)	Without SOD (tube4-6)	Effect of SOD p	With SOD (tube3-5)	Without SOD (tube4-6)	Effect of SOD p
All	0.009(0.042) n=18	-0.026(0.076) n=21	0.052	0.013(0.064) n=18	0.012(0.101) n=22	0.987
Diabetics	0.007(0.045) n=13	-0.039(0.084) n=15	0.064	0.006(0.073) n=13	0.021(0.098) n=16	0.771
Non-diabetics	0.015(0.029) n=5	0.004(0.046) n=6	0.622	0.031(0.033) n=5	-0.004(0.073) n=6	0.232
	*p=0.818	*p=0.277		*p=0.481	*p=0.586	

Table 4.3: The absorbance of ferricytochrome C (Fc) attributable to platelet aggregation in the presence and abscence of superoxide dismutase (SOD).

Results are the mean (SD) differences in OD between ferricytochrome C exposed to aggregating and resting platelets ie. the effect attributable to aggregation. A negative value suggesting less ferricytochrome reduction in the presence of aggregation.
*p refers to the significance of the comparison between diabetics and non-diabetics.

production in association with resting platelets. This superoxide production was not increased by aggregation, questioning its putative platelet origin. Possibly washing fails to separate platelets from the active plasma components as efficiently as gel filtration. The difference in the results of these studies was not due to the loss of function of gel filtered platelets as their viability was confirmed by their ability to aggregate in response to collagen.

The possibility that $O_2^{\bullet-}$ was released but took part in other kinetically more favourable reactions, thereby remaining undetected, is unlikely. Gel filtration would have removed most of the plasma components such as lipids and proteins which would have been capable of undergoing such reactions. $O_2^{\bullet-}$ can undergo spontaneous dismutation in aqueous solution (Equation 4.2) but the rate is pH dependent and greater at low pH and so less likely at the physiological pH used here where the rate constant is 5×10^5 $M^{-1}s^{-1}$ (13).

Equation 4.2 $$O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$$

The reaction catalysed by superoxide dismutase is pH independent in the pH range 5.3-9.5 and has a rate constant around 3,000 times that of the spontaneous reaction (1.6×10^9 $M^{-1}s^{-1}$). The rate constant for the reaction of $O_2^{\bullet-}$ with Fc (Equation 4.1) is (1.1×10^6 $M^{-1}s^{-1}$) (242) and more nearly approximates to spontaneous dismutation but the concentration of Fc (2×10^{-4} M) is so greatly in excess of that of the H^+ concentration (10^{-7} M) that reaction with the former would be favoured. Theoretically lipid peroxides and other secretory products of platelets may be capable of reaction with Fc causing its re-oxidation and so masking reduction. As no overall effect was observed, for this to be the explanation, the release of exactly equivalent amounts of oxidising and reducing species would have to be postulated but seems highly improbable.

Because Fc was present in the reaction mixture before the addition of platelets it is not possible that a burst of $O_2^{\bullet-}$ release occurred prior to its addition and was missed.

I employed physiological concentrations of platelets in this study, and it is possible that the assay was not sensitive enough to detect the small resultant concentration of $O_2^{\bullet-}$. Platelet concentration at sites of injury may be much higher and larger concentration of $O_2^{\bullet-}$ may be produced in those circumstances.

In agreement with Marcus *et al* (241) I observed no inhibition of platelet aggregation by SOD or Fc. However these results may seem to disagree with those of Salvemini

et al (243) who reported inhibition of thrombin induced adhesion by both substances and inhibition of aggregation by SOD. However this effect was seen only at concentrations of thrombin producing less than 45% aggregation, and was lost at higher concentrations producing greater percentage aggregation. In the current study collagen was used and at doses achieving greater than 70% aggregation and so actually in agreement with Salvemini *et al*, no inhibition was observed. Salvemini *et al* concluded from the inhibition seen at low thrombin dose that $O_2^{\bullet-}$ was involved in platelet activation. The results here do not contradict that conclusion. I looked at the reduction of Fc in the supernatant surrounding platelets, the platelets were removed by centrifigation prior to measurements of OD, and no reduction of Fc was observed therefore it can be concluded only that $O_2^{\bullet-}$ was not released to the external environment of aggregating platelets, not that it has no involvment in activation. Salvemini *et al* did not offer an explanation as to why the effect of SOD on platelet aggregation is lost at high doses of thrombin. An explation of that phenomenon may also offer another reason why I observed no effect of SOD on collagen induced aggregation. The set of reactions leading to aggregation induced by one agonist is similar to but different from that induced by other agonists. Some agonists including thrombin have modes of action dependent upon their concentration (244). Therefore it is possible that $O_2^{\bullet-}$ dependent reactions are involved in the aggregation induced by one concentrtion of thrombin but not by another, and by low dose thrombin but not collagen.

The lack of $O_2^{\bullet-}$ release by platelets excludes only one possible mechanism of platelet enhanced oxidation of LDL. Platelets have LDL receptors and $O_2^{\bullet-}$ produced by an enzyme system located within the cell membrane may not need to escape it to react with bound LDL. They may release other radicals (239) or oxidise LDL by other means. Aviram *et al* (236) has reported that the medium surrounding activated platelets (platelet conditioned medium, PCM) enhanced copper stimulated LDL oxidation by a mechanism which involved FR, but since PCM alone did not oxidise LDL it did not appear to contain the oxidising species *per se* but enhanced their production in an unidentified way (236).

4.7. CONCLUSION.

This study does not support the hypothesis that platelets at rest or during aggregation release superoxide anion. This is true for diabetic and non-diabetic subjects. The

proposal that superoxide anion release by platelets may be a mechanism whereby platelets contribute to the generation of atherosclerosis is also unsupported by these findings. However, only the effect of one agonist, collagen, was studied and work with other agonists may be valuable. It has not been excluded that platelets enhance LDL oxidation by other mechanisms and other interactions between platelets, macrophages and lipoproteins not involving LDL oxidation may prove to have more important roles in atherogenesis (236, 245, 246)

CHAPTER FIVE

PLASMA ANTIOXIDANTS IN DIABETES MELLITUS.

5.1 INTRODUCTION

Since FR oxidation of LDL may increase its atherogenicity then mechanisms that impede FR reactions might be expected to protect LDL from oxidation and therefore be anti-atherogenic. Diabetic subjects are at increased risk of atherosclerotic disease and I wished to ascertain if this might be due in part to impaired FR defences.
This chapter and the next deals with a single study designed to test two hypotheses. These hypotheses are :-

1. Plasma total antioxidant activity, as measured by TRAP, is lower in diabetic subjects than in healthy controls, thereby exposing diabetics to increased risk of FR mediated damage.

2. The extent to which the lipids in plasma from diabetic subjects peroxidise will be inversely related to plasma total antioxidant activity, as measured by TRAP.

This chapter describes the study and deals with the background, results and discussion pertaining to the first of these hypothesis including a discussion of the rational for the use of the relatively novel TRAP assay to assess FR defences. For the sake of clarity, the second hypothesis is dealt with in chapter six.

5.1.1. The Nature Of Free Radical Defence Mechanisms

Because they involve single electron transfer, any reaction between a radical and a non-radical inevitably results in the formation of a new radical. Hence FR reactions, once initiated, progress via an auto-catalytic chain reaction. Radicals are annihilated only on reaction with other radicals. Undesirable FR processes are limited *in vivo* by many strategies; some are aimed at preventing initiation of the chain, others at its interruption. Some are specific, others less so.

a.Preventative Defence Mechanisms.

Non-specific, preventative strategies of control include the fact that FR intermediates, produced in the course of normal metabolism, are generally securely enzyme bound restricting their reactivity. Also, compartmentalisation of metabolic processes means that products of one pathway, including FRs, may have limited effects on other pathways. Another non-specific method of control involves binding of transition metals (17, 201). As mentioned earlier, copper (Cu^{+}) and iron (Fe^{2+}) ions are capable of catalysing FR chain reactions and reactions which result in the generation of FRs (Fig 1.2). Few, if any, of these ions are left free but form complexes with proteins such as caeruloplasmin, transferrin and ferritin and low molecular weight molecules such as ATP. This provides restriction of their catalytic potential although there is some debate as to the efficiency of that restriction in certain circumstances (15, 247). It has been noted that EDTA-Fe complexes do support $OH^{\cdot}$ production *in vitro* and this raises the possibility that other low molecular weight, non-protein transition metal complexes may be active *in vivo* (247, 248). Also, it was found that the iron bound in lactoferrin and transferrin could be mobilised, and so was capable of catalysing $OH^{\cdot}$ production when the limit of the Fe^{2-} binding capacity of these proteins was approached, and/or the pH reduced (247). In addition, $O_2^{\cdot-}$ was found to reduce and release iron from ferritin implying that vicious cycles of metal ion release and FR production could arise (249). Evidence that such iron mobilization does occur and can be pathologically relevant *in vivo* comes from animal studies showing that FR production and damage during ischaemia and reperfusion were inhibited by the iron chelator, deferoxamine (23). Incomplete restriction of transition metal catalysed FR activity may also have a role in diseases of iron overload such as rhesus disease and thalassaemia (250). In addition to simply binding copper, caeruloplasmin also has a ferroxidase activity, oxidising Fe^{2+} to Fe^{3+} (251). In this valency state, iron can no longer catalyse the Haber-Weiss reaction, which produces $OH^{\cdot}$ (15). All of these methods achieve control of FR processes by preventing the initiation of harmful chain reactions. The same method underlies the more specific effect of catalase and the peroxidases, including glutathione peroxidase. By removing H_2O_2 and other peroxides, which can be precursors of FRs (Fig 1.2), new radical chain reactions are prevented (26, 219).

b. Chain Breaking Defence Mechanisms.

Mechanisms in which the "antioxidant" reacts directly with, and disarms, an already formed radical may inhibit, not the initiation of the chain reaction, but its progression. Mechanisms employing this "chain-breaking" approach are of several types (26) but include the enzymic activity of superoxide dismutase (SOD). SOD specifically removes $O_2^{\cdot-}$ by catalysing its reaction with a second $O_2^{\cdot-}$ ion causing their dismutation to H_2O_2 (24).

Other chain breaking antioxidants are non-enzymic and can be divided into two groups. The first group are molecules generated endogenously for other purposes but also capable of antioxidant activity such as uric acid, bilirubin, glutathione and the thiol groups of amino acid side chains in proteins (13, 26). The second group cannot be synthesised *in vivo* by man and are provided only by the diet and include vitamins C, E, A and the pro-vitamin β-Carotene (17). In the terminology, as applied to fatty and amino acids, these antioxidants might be thought of as non-essential and essential respectively.

In the process of radical removal, these non-enzymic chain-breaking antioxidants are themselves chemically altered leaving them not only incapable of further antioxidant activity but also, at least temporarily, converted to another radical. On first inspection the process seems to offer little advantage, merely substituting one radical for another, whilst losing the antioxidant. Such sacrificial loss initially appears extravagant, especially in the case of essential antioxidants. However, not all radicals are of equal potency (17, 201) and the new radical is usually one of lesser reactivity, and therefore less capable of damaging macromolecules. Also, there frequently exist mechanisms for recycling the antioxidant. For example, the active, antioxidant form of vitamin C is the reduced, non-radical ascorbic acid (AA) (252). It is oxidised during reaction with such radicals as $O_2^{\cdot-}$ and $OH^{\cdot}$, becoming the ascorbate radical, semidehydroacsorbate, (SDHA) (Fig 5.1). Although a radical, SDHA is less reactive than $O_2^{\cdot-}$ or $OH^{\cdot}$. Recycling begins with the reaction of two SDHA radicals which results in the formation of one AA molecule and one dehydroascorbic acid (DHA), the latter being neither a radical nor an antioxidant (Fig 5.1) (13, 119). In addition DHA is then recycled to AA by a glutathione-dependant reductase (Fig 5.2) (13).

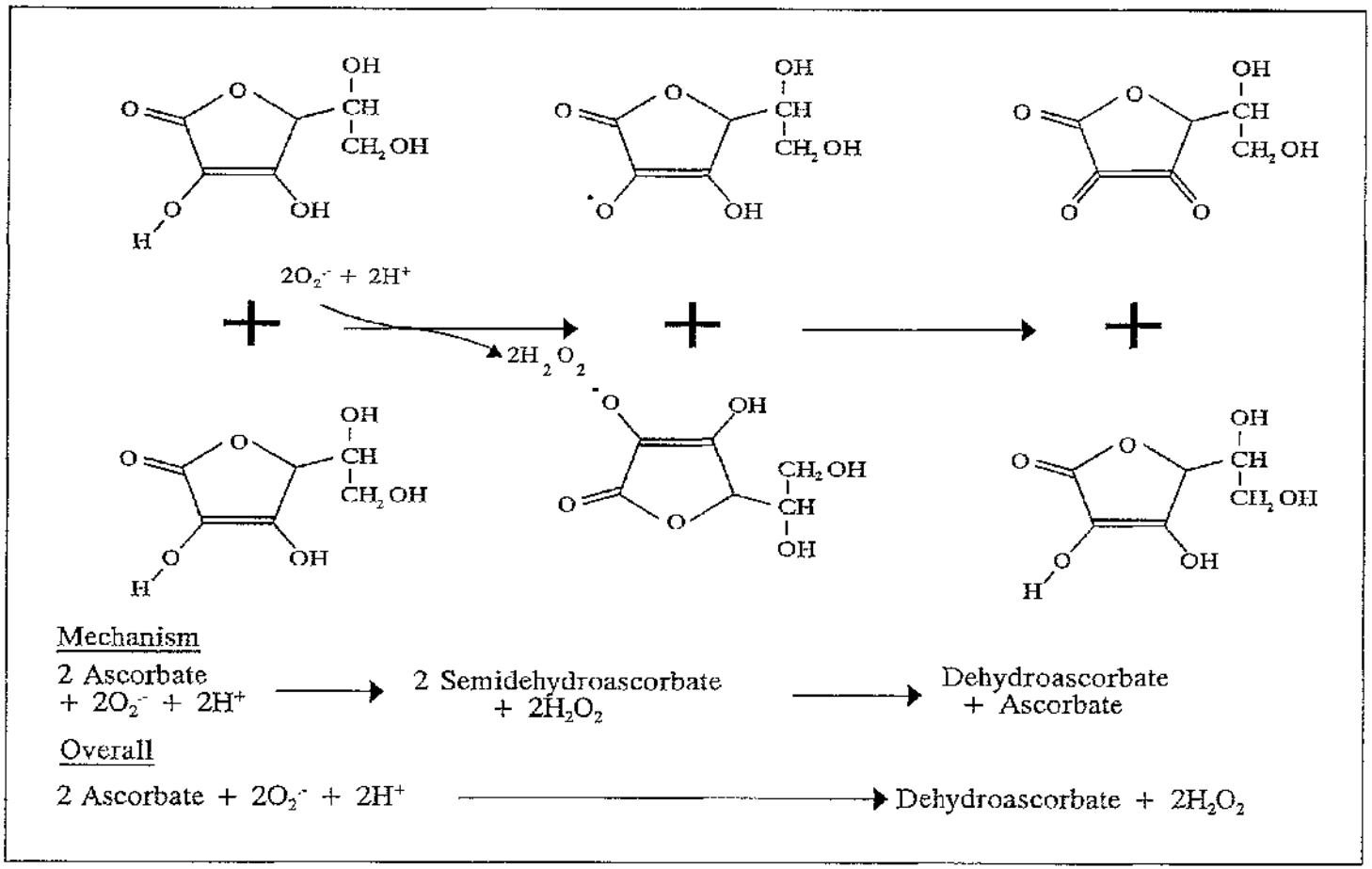


Figure 5.1: Ascorbate as a scavenger of superoxide radicals.

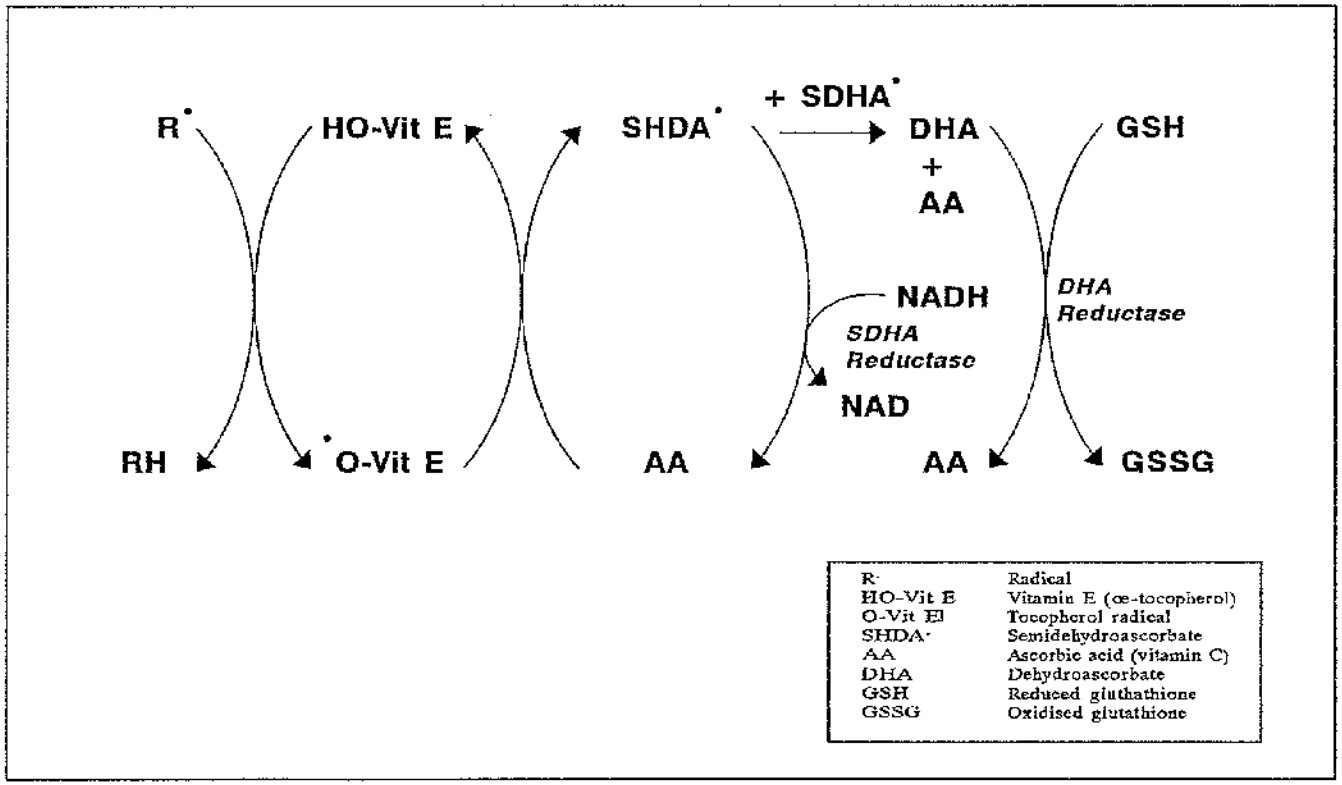


Figure 5.2: The recycling of, and interaction between, vitamin E and C (ascorbic acid) during free radical scavenging.

5.1.2. Clinical Relevance Of Free Radical Defence Mechanisms In Atherosclerosis

As already discussed, FR oxidation of LDL may be important in the genesis of atheroma. Such oxidation would become more probable in situations in which FR defence mechanisms were impaired. Hence these mechanisms may be of relevance pathologically and a knowledge of them, and how they might come to be abnormal, may add to our understanding of the pathogenesis of atheroma.

These mechanisms may also be of therapeutic importance. Should the oxidation theory of atheroma prove correct, manipulation of these mechanisms may offer means of treatment and protection against atheromatous vascular disease. The mechanisms influenced by diet, such as those involving the essential chain-breaking antioxidants, are likely to be the most readily manipulable. Some encouragement for this way of thinking has come from studies on probucol. Probucol is a drug prescribed to lower plasma cholesterol. However, it is structurally similar to the antioxidant butylated hydroxytoluene and also has antioxidant properties. *In vitro* it has been shown to reduce cellular and metal catalysed oxidation of LDL (253). *In vivo,* when compared to doses of lovastatin which produce similar degrees of cholesterol lowering, probucol also reduced the progression of atherosclerotic lesions in Wantinabe heritable rabbits (254). These animals are deficient in the LDL receptor and serve as a model for Familial Hypercholesterolaemia. This was taken to imply that probucol's anti-atherogenic action was not simply the result of its hypocholesterolaemic effect and suggested the possibility that its antioxidant properties were responsible. It has to be borne in mind of course that probucol may have yet other properties, currently unidentified, accounting for this activity.

Like Probucol, ascorbic acid and α-Tocopherol, the most active form of vitamin E, reduce cellular and metal catalysed human LDL oxidation *in vitro* (28, 255). Also it has been reported that LDL isolated from humans having taken vitamin E supplements displayed resistance to oxidation *in vitro* (46). Dietary supplements of vitamin E which raised plasma levels four fold attenuated atherosclerotic lesion development in Watanabe rabbits (256). These studies suggest that the naturally occurring antioxidants, vitamins E and C, might also have anti-atherogenic effects. It is also possible, but cannot be assumed, that they would have the advantage over probucol of fewer side effects. Hence pathological insights and therapeutic possibilities may be gained by assessment and greater understanding of FR defence mechanisms.

5.1.3. Antioxidants In Diabetes And Ischaemic Heart Disease

Many abnormalities of antioxidant levels have been reported in diabetes and were outlined in section 1.3.2. Abnormalities have also been described in ischaemic heart disease. In a case control study, lower plasma levels of vitamins E and C were found in a group of men suffering from angina. Whereas the relationship between low vitamin C levels and the risk of angina was confounded by smoking, this was not true for vitamin E levels. Further, low vitamin E levels remained related to risk of angina even after allowing for age, blood pressure, total and HDL cholesterol, triglycerides and weight (199). This suggests an independent, inverse relationship between vitamin E levels and risk of angina. Whether low vitamin E is a cause or a consequence of angina cannot be concluded from such a relationship, however the situation is made a little clearer by cross cultural, population studies of apparently normal volunteers in which vitamin E and C levels were found to be lower in groups from areas associated with high risk of cardiovascular disease compared to those from areas associated with low risk (198). If reduced levels predate disease they cannot be a consequence and is more consistent with, but still not proof of, a causal relationship.

5.1.4 Problems In Interpreting Abnormalities In Antioxidant Levels.

Thus, many abnormalities in antioxidant levels have been noted in those suffering from diabetes and cardiovascular disease. However, so little is known of how antioxidant levels are controlled *in vivo* that interpretation is not straightforward. As always, there is the problem as to whether the abnormality is "cause or consequence" of the disease process or an epiphenomenon. In addition, because of the close links between antioxidants and FRs, antioxidant levels are frequently interpreted in terms of FR activity and are even used as indirect markers of FR activity. However, this requires great caution as reduced antioxidant levels might suggest, for example, greater consumption due to increased FR production, but it is also possible that reduced antioxidant activity is the body's response to reduced demand, so reflecting reduced FR production. This type of argument was used by Jones *et al* who found, not reduced levels of caeruloplasmin and iron binding proteins, in a group of diabetic subjects, but increased levels (251) and yet they were able to conclude that this was still compatible with increased FR activity in diabetes

and reflected a response to that activity. Despite the difficulties with interpretation, there are obviously many studies which do suggest that FR defense mechanisms are perturbed in atherosclerosis and diabetes, although there is some doubt as to whether antioxidants are reduced as a consequence of the disease or increased as a response. Another difficulty with interpreting antioxidant levels in terms of FR activity is that FR activity is not the only factor affecting antioxidant levels. Diet and smoking may both have major influences on antioxidant intake and survival (169, 195, 197). Also age has been reported to be associated with alterations in antioxidant enzyme systems (206), vitamin A (257) and vitamin E cocentrations (258).

5.1.5. Problems In Measuring Antioxidant Levels.

As in the studies mentioned above, FR defence mechanisms are often assessed by measuring the concentration of one or more individual antioxidants. It is often assumed that those measurements reflect overall antioxidant status and FR defences. Although stimulating information has been gained by this approach it does have problems, even in addition to those of interpretation. These arise because the protective activities do not work in isolation but in consort (26). This allows for the possibility that a low level of one may be compensated for by elevated levels of another, and therefore an alteration in a single antioxidant may not reflect overall antioxidant status. One way around this difficulty would be to measure the concentration of all antioxidants and, using the stoichiometry of their reactions with radicals, calculate the total antioxidant activity (260). This is limited in many ways. Firstly, not all antioxidants are known. Secondly, the stoichiometry of their reactions with radicals is debated (26), and may differ depending on the radical involved, but also depending on the environment in which the reaction occurs and the concentration of the reactants (261). In addition, complex interactions between antioxidants occur for example the vitamin E radical formed during FR scavenging is reactivated by interaction with vitamin C (Fig 5.2) (262). In its turn vitamin C is reactivated as described above in section 5.1.1. Such interactions mean that simple summation of the calculated activities based on concentrations of individual antioxidants would be misleading (260).

Some integrated measure of activity would be of greater value. Wayner *et al* have described such a method (127) and it is employed in this work and it measures the total peroxyl radical trapping antioxidant parameter (TRAP) of plasma in what is

almost a bioassay. The principle behind this assay is outlined in the next section. It makes no assumption regarding the nature or concentration of individual antioxidants in plasma and its value will be contributed to by all species capable of reacting with peroxyl radicals, including vitamins C, E, uric acid etc. The fact that it relates to the peroxyl radical makes this method particularly appropriate for the study of LDL lipid oxidation because, as was discussed in chapter 3, oxidation of the lipid, once initiated, is propagated by peroxyl radicals (Fig 3.1).TRAP gives an estimate of antioxidant equivalence, as opposed to a concentration, since the stoichiometry of reactions between antioxidants and radicals is not one to one.

5.1.6. Principle Of The TRAP Assay.

The precise methodological details of this assay are given in chapter two (Section 2.3.6.). Plasma contains antioxidants and their combined effects confer plasma with the ability to inhibit oxidative processes. In the TRAP assay plasma is used to inhibit the peroxidation of linoleic acid and the extent of this ability is determined by comparing it with that of a known amount of antioxidant (Figs 5.3 and 5.4). In the experiment, linoleic acid is mixed with, and peroxidised by exposure to, 2,2'-azo-bis(2-amidinopropane) hydrochloride (ABAP) which thermally decomposes with the release of peroxyl radicals (Fig 5.3) (127). The peroxidation of linoleic acid is accompanied, and so can be followed, by uptake of oxygen (Fig 5.3). The peroxidation of linoleic acid, and so the uptake of oxygen, in the presence of plasma begins only after a time lag. A typical O_2 uptake curve is shown (Fig 5.4). The duration of this time lag, or induction phase (T_p), is determined by the antioxidants in plasma. Addition of a known amount of antioxidant also produces an induction phase (T_a) . By comparing T_p and T_a the antioxidant capability of plasma can be calculated. In this study the antioxidant used was Vitamin C and a stoichiometry of its reaction with peroxyl radicals was taken to be 1.7 (26).

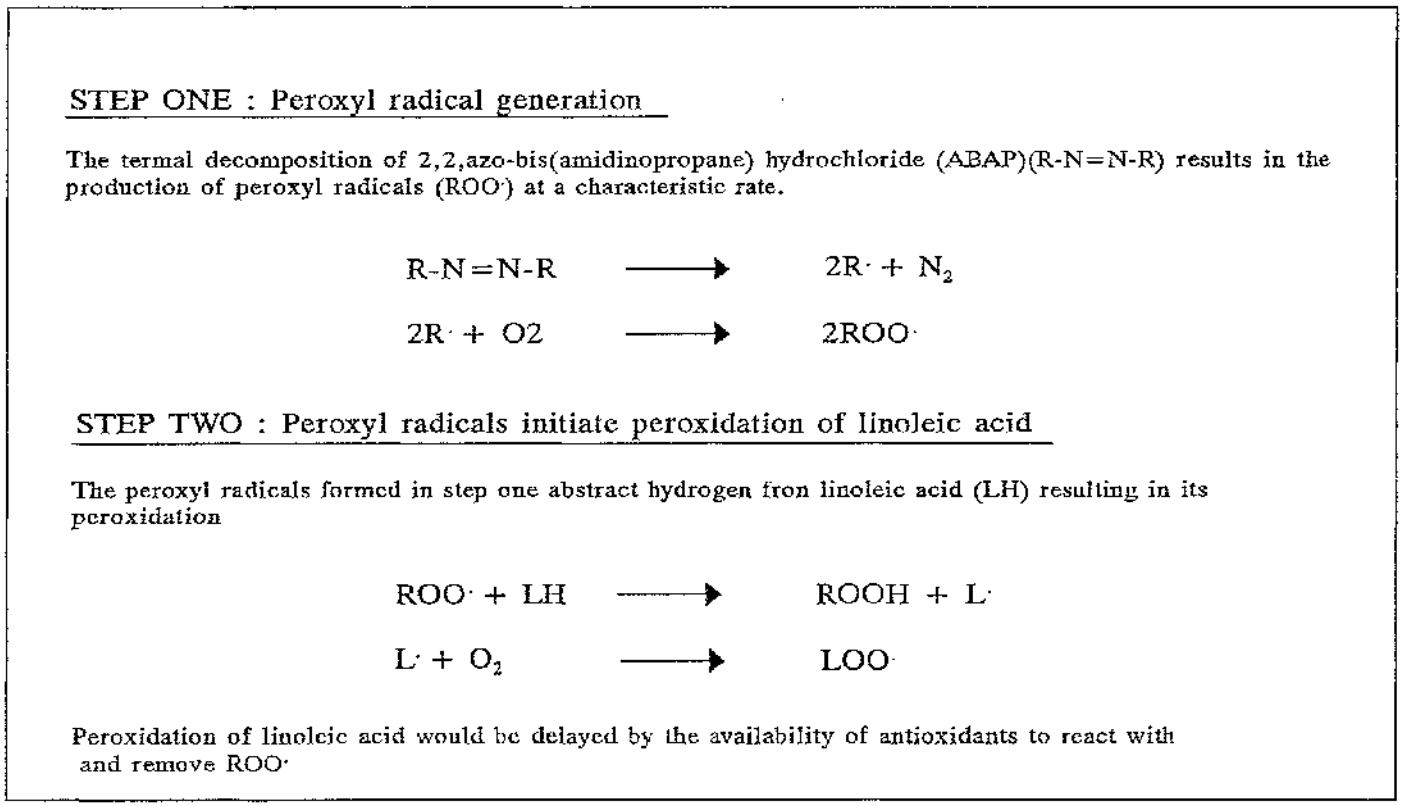
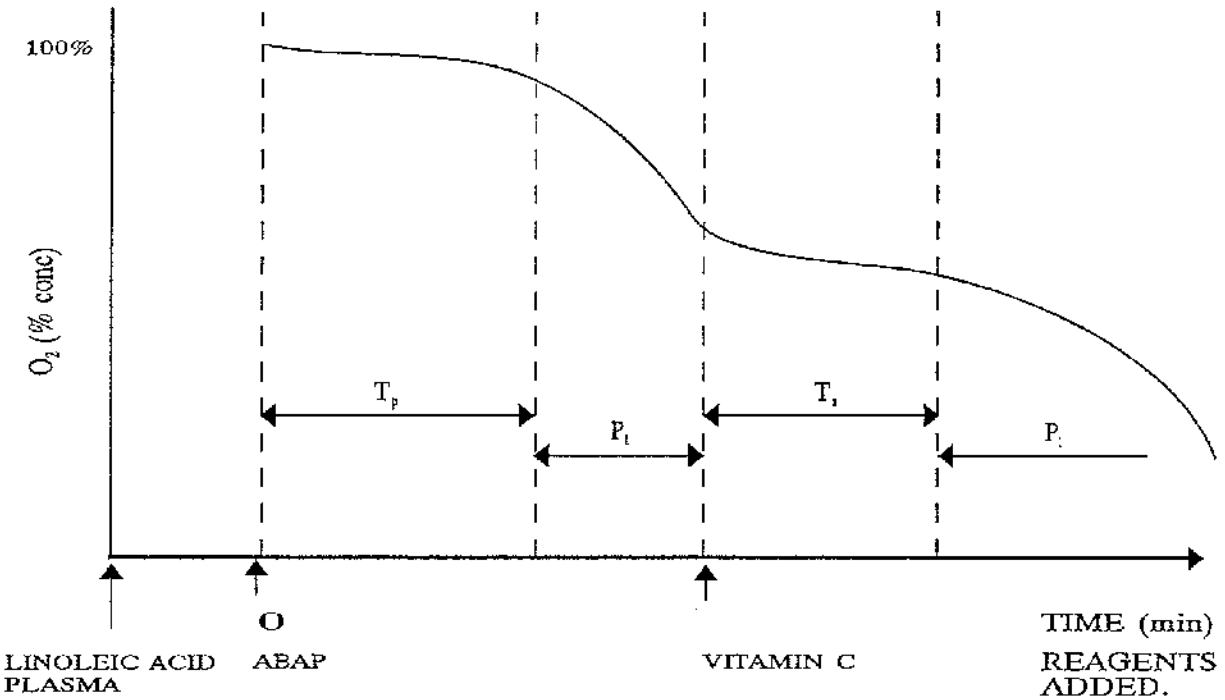


Figure 5.3: TRAP assay principle.



The times, T_p and T_t, are measured from the trace and used as described in section 5.6.1. to calculate the total peroxyl radical antioxidant activity of plasma (TRAP).
Where ABAP is 2,2'-azo-bis(2amidinopropane) hydrochloride

Figure 5.4: A representation of an oxygen uptake trace as generated during the TRAP assay

The equation for calculation of TRAP is derived as follows:-

Since:-

$$\frac{T_p}{T_a} = \frac{[\text{"conc."}\ \text{Antioxidants in plasma}] \times \text{Vol. plasma}}{[\text{conc. of Ascorbate}] \times n \times \text{Vol. Ascorbate}}$$

where:-

n = the number of Peroxyl radicals trapped per ascorbate molecule = 1.7

["conc." of antioxidant in plasma] = TRAP

Then:-

$$\text{TRAP} = 1.7 \times [\text{conc. Ascorbate}] \times \frac{T_p}{T_a} \times \frac{\text{Vol. Ascorbate}}{\text{Vol. plasma}}$$

where :- [conc. Ascorbate] = $500\mu mol.l^{-1}$

Vol Ascorbate = $25\mu l$

Vol plasma = $50\mu l$

$$\text{TRAP} = 425 \times \frac{T_p}{T_a}$$

5.1.7. Summary

Abnormalities in antioxidants may influence the genesis of a more atherogenic LDL. Such abnormalities have been reported in diabetes and may help to explain the greater incidence of atherosclerotic problems in diabetic patients. However, these reports derived from studies making use of arguably simplistic measures of antioxidant status. I wished to employ a method, namely the TRAP assay, which theoretically determines antioxidant status more accurately and would therefore give a more clearcut impression of whether or not this status is abnormal in diabetes. The problems of how to interpret alterations in antioxidant status remain.

5.2. HYPOTHESIS.

Plasma total antioxidant activity, as measured by TRAP, is lower in diabetic subjects than in healthy controls, thereby exposing diabetics to increased risk of FR mediated damage.

5.3. SUBJECTS AND STUDY DESIGN

TRAP values were established in seventeen non-diabetic healthy volunteers; their characteristics are given in Table 5.1. Diabetic subjects were recruited from among the routine diabetic clinic attenders. Patients attending the clinic were asked to come "fasting", pre-breakfast or pre-lunch, depending on the time of day of the clinic, hence having fasted for more than 3 hours. All patients attending the clinic had venous blood drawn for estimation of plasma glucose and HbA_{1c}. Patients were approached by the investigator (MEB) who was blind to their clinical details. Patients were excluded if not "fasting" otherwise each subject was asked to allow an extra 20mls of blood be taken. All eligible subjects agreed. Clinical details were subsequently determined by questioning the patients and supplementing their histories by reference to the hospital notes. Ten subjects were recruited in this way on each of three days within 4 weeks. Their clinical details are are given in Table 5.1. Peripheral vascular disease, ischaemic heart disease, nephropathy and neuropathy are defined as in the methods section. Subsequently technical difficulties made

	Diabetic Subjects	Non-diabetic Subjects	p Value.
Number (n)	30	17	
Age (yrs)	60.9(16.2)	42.2(9.5)	< 0.001
Smoking (Yes:No)	11:19	4:13	> 0.2# (χ^2=0.97)
Sex Ratio (M:F)	14:16	6:11	>0.2# (χ^2=0.44)
Duration (yrs)	9.0(3.0,12.0)		
Type of diabetes (IDDM:NIDDM)	8:22		
Treatment			
Diet	7	N/A	
Sulphonylurea	6	N/A	
Sulphonylurea plus metformin	8	N/A	
Metformin	1	N/A	
Insulin	8	N/A	
Complication			
None	12	N/A	
Microvascular	11	N/A	
Macrovascular	2	N/A	
Micro plus macro	3	N/A	
Hypertension	2	N/A	
HbA_{1c} (%) (NR=6.5-8.5)	10.9(2.2)	6.4(0.6)	0.0001
Fasting plasma glucose (mmol l^{-1})	10.5(4.6)	5.1(0.6)	0.0001
TRAP (μmol l^{-1})	661.8(173.8)	774.3(103.7)	0.01

Table 5.1: Clinical details of subjects.

N/A : Not applicable.
NR : Normal range.
Values are given as mean (SD) or median (25th, 75th percentile). Comparisons between groups are tested using unpaired t-tests or chi-squared test#

estimation of TRAP in four diabetic subjects impossible therefore results are presented for twentysix subjects.

Since alterations in certain antioxidants believed to contribute to TRAP have been related to age and smoking these features were noted and included in the statistical analysis. The possible contribution of duration of diabetes and of metabolic control on reduced antioxidant status were assessed by measurement of HbA_{1c} and by recording the time since diagnosis of diabetes, incorporating this data into statistical analysis.

As the second limb to this study "peroxidisability" of plasma lipids was measured in the diabetic subjects and this aspect of the study is dealt with in chapter six.

5.4. METHODS.

The analytical and stastistical methods used are described in chapter two.

5.4.1. Assays Performed.

Fasting plasma glucose (FPG)
Glycosylated haemoglobin (HbA_{1c})
Total cholesterol (total-Chol)
Triglyceride (TG)

Total Peroxyl Radical Trapping Activity of Plasma (TRAP)

Plasma peroxidisability - these results will be discussed in chapter six ,and it was measured in the diabetic subjects only.

5.5 RESULTS

The age of control subjects was significantly less than that of the diabetic group (42.4(9.5) vs 60.9(16.2)years, $p=0.001$) but age did not correlate significantly with TRAP ($r=0.017$, $p=0.915$) (Fig.5.5). The proportion of smokers in the diabetic group was not significantly different from that in the controls ($\chi^2=0.97$, $df=1$, $p=0.2$) and the TRAP in smokers was not significantly different from that in non-

smokers (638.4(200.7) vs 744.9(123.3)μmol.l^{-1}, p=0.09) (Fig.5.6). The TRAP in diabetic subjects was found to be lower than in non-diabetic controls (661.8(173.8) vs 774.3(103.7)μmol.l^{-1}, p=0.01) (Fig 5.7) however using analysis of variance with both diabetes and smoking as independent variables a significant interaction between diabetes and smoking was suggested (Table 5.2). Therefore separating the subjects into diabetic and non-diabetic smokers and non-smokers revealed that in non-smokers TRAP was not significantly different in diabetic and non-diabetic subjects (736.5(151.8) vs 754.6(84.3)μmol.l^{-1},p=0.70) whereas in smokers TRAP was lower in diabetics than in non-diabetics (558.5(161.5) vs 838.3(147.5)μmol.l^{-1}, p=0.02). Also in diabetics TRAP was significantly lower in smokers than in non-smokers (558.5(161.5) vs 736.5(151.8)μmol.l^{-1}, p=0.01) (Fig 5.8). The inclusion of age in the analysis of variance model with diabetes and smoking confirmed that age did not significantly influence TRAP (Table 5.2).

Within the diabetic group, TRAP did not correlate with age, duration of diabetes (Fig 5.9), fasting plasma glucose nor HbA_{1c} (Fig 5.10) in univariate analysis (Table 5.3). In a multiple linear regression model these relationships remained non-significant (Table 5.4a). This remained true when smoking was included in the model which also confirmed that smoking was a significant determinant of TRAP in diabetics (Table 5.4b)

TRAP levels did not significantly differ between those with and without complications nor between those with type 1 or type 2 diabetes nor between the sexes (Table 5.5).

5.6. DISCUSSION

5.6.1. TRAP And Diabetes.

Although age was different in the diabetic and non-diabetic subjects studied it was not found to be correlated with TRAP in this study, in univariate or multivariate analyses, and so I will not discuss it further in terms of confounding effects upon TRAP in diabetes. However, as previously mentioned, age has been associated with lower levels of certain individual antioxidants (206, 257, 258). The fact that I find overall antioxidant activity not negatively correlated suggests th epossibility that with ageing a new balance of individual antioxidants is reached with some falling whilst others rise hence acheiving no overall change. In keeping with this possibility a

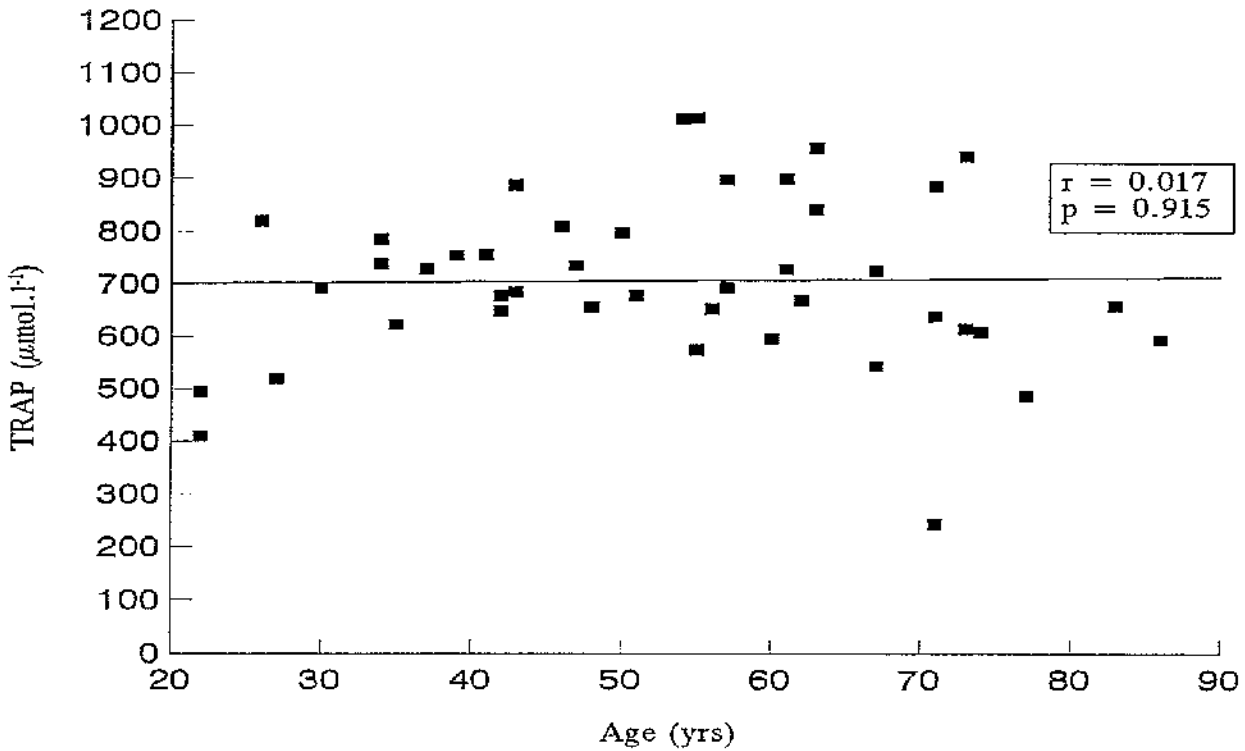


Figure 5.5: The correlation between TRAP and age

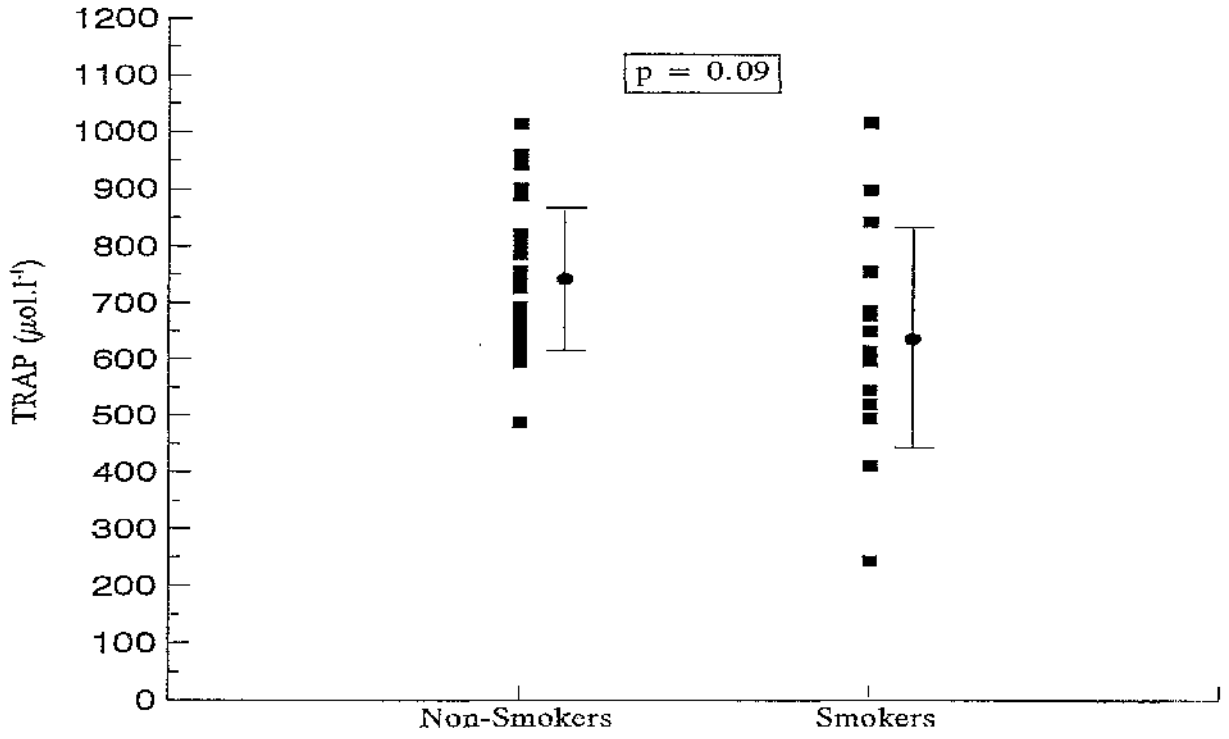


Figure 5.6: TRAP in non-smokers and smokers

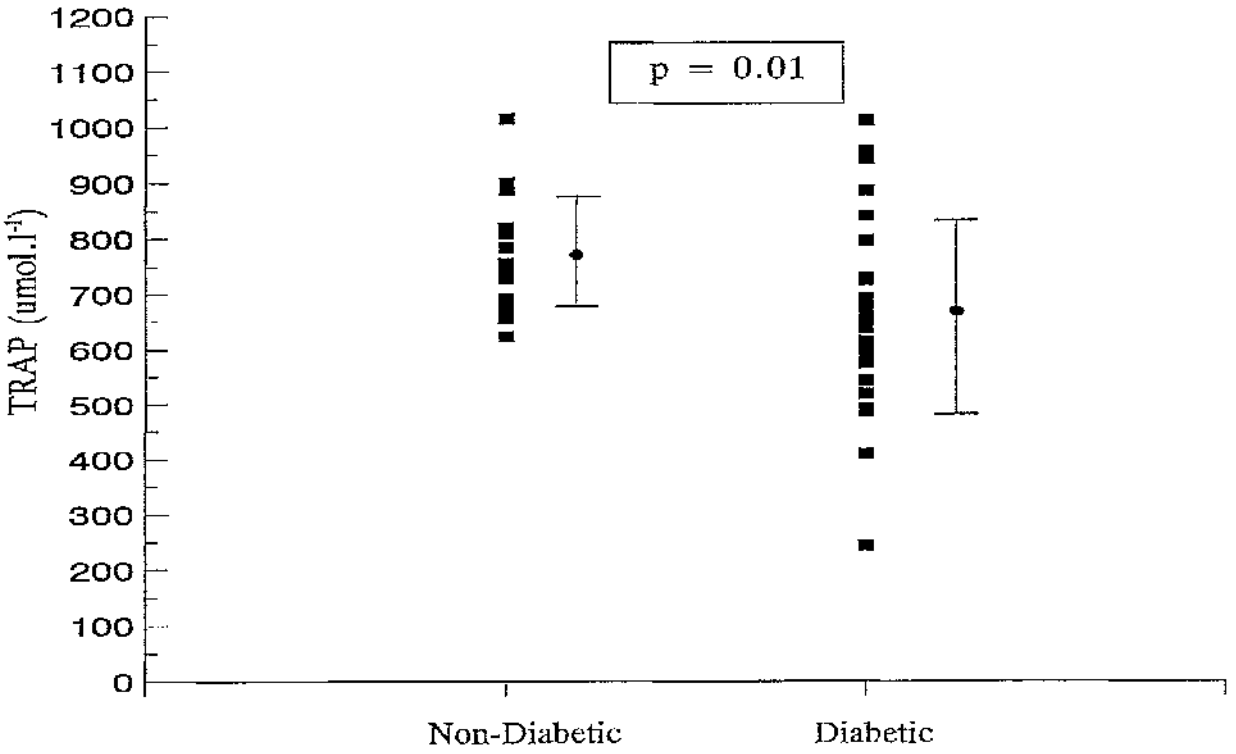


Figure 5.7: TRAP in non-diabetic and diabetic subjects

Dependant variable	Source of variation	F value	p value
TRAP	Age	0.21	0.887
	Presence of diabetes	5.993	0.019
	Smoking	2.342	0.134
	Interaction of diabetes and smoking	5.791	0.021

Table 5.2: The effect of smoking, the presence of diabetes and age upon TRAP.

The analysis of variance table showing the variance ratios (F) explained by the listed variable and the associated level of significance (p).

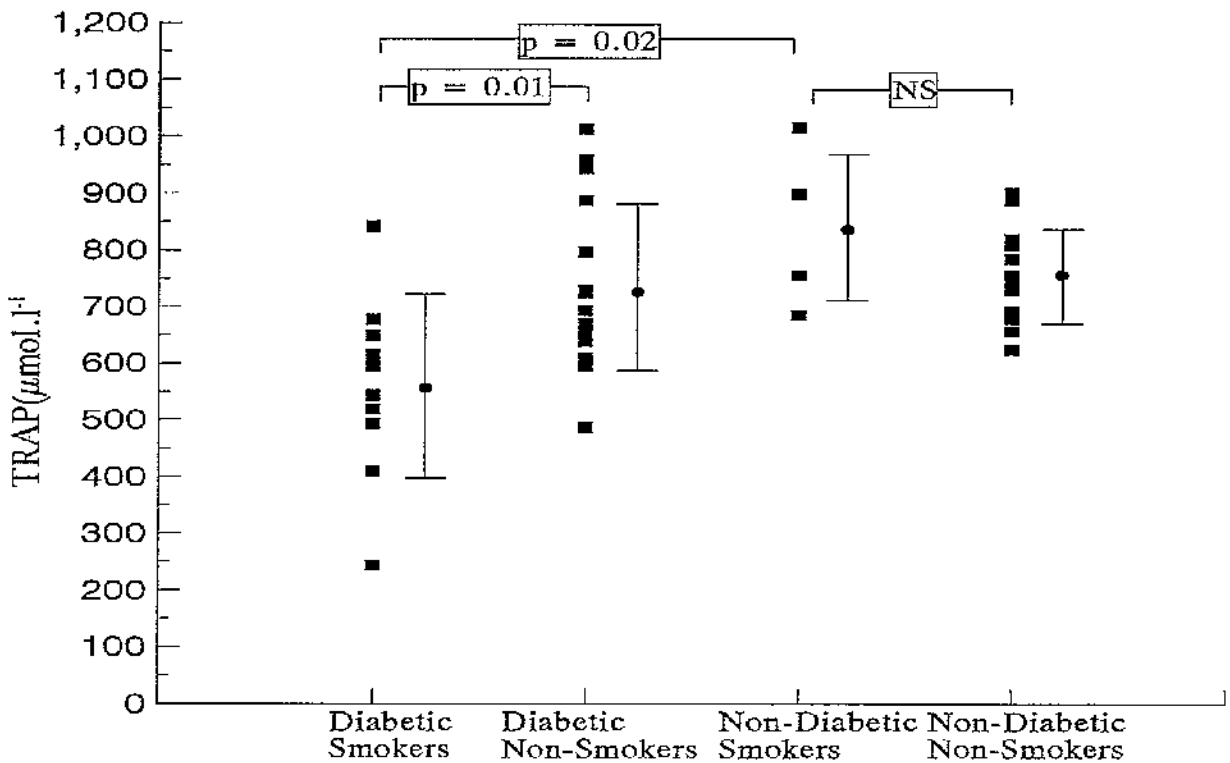


Figure 5.8: TRAP in relation to diabetes and smoking.

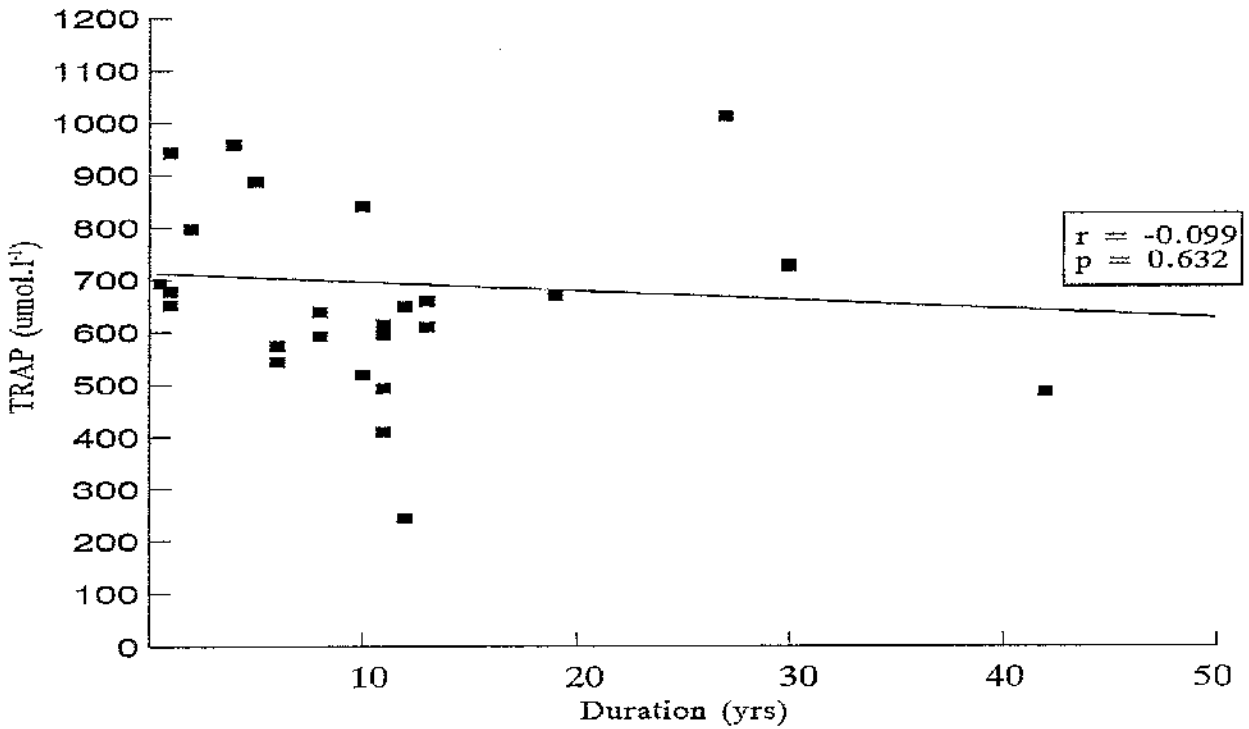


Figure 5.9: The correlation of TRAP with the duration of diabetes.

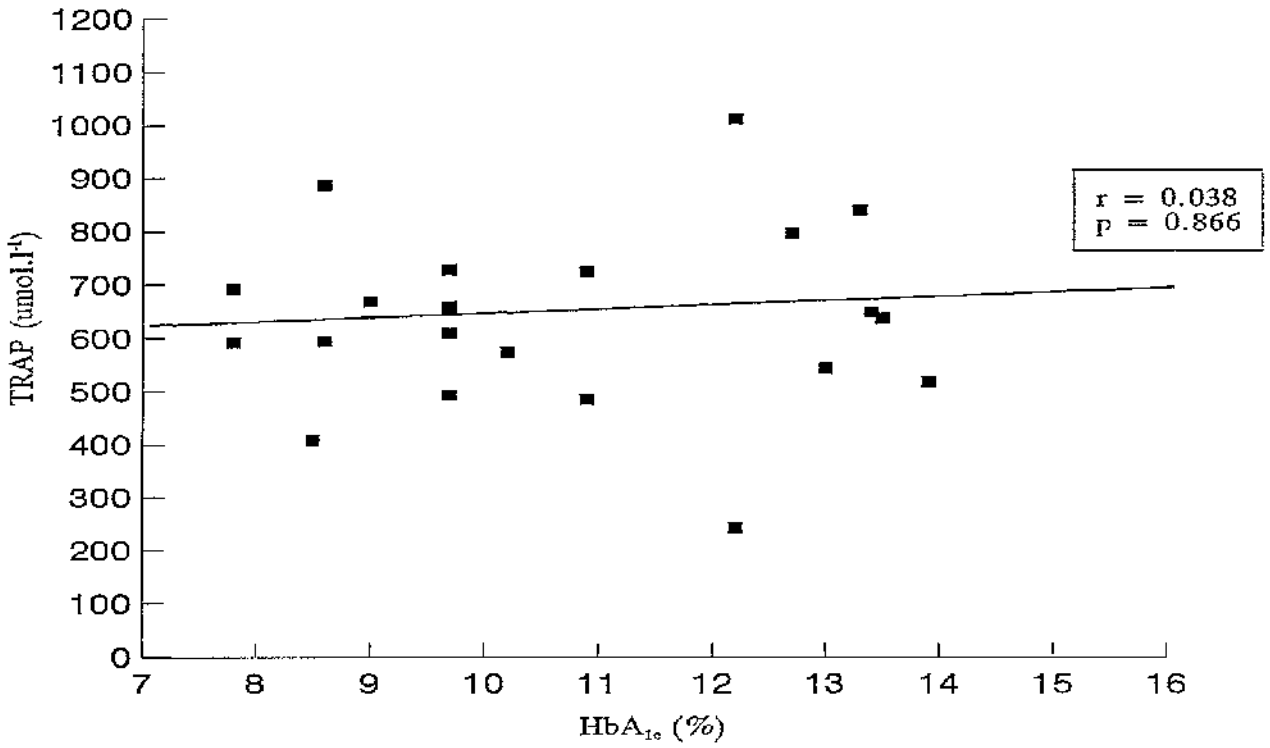


Figure 5.10: The correlation of TRAP with HbA_{1c}.

		TRAP	
		Correlation Coefficient	p
Age (yrs)	n=26	0.190	0.354
Duration of diabetes (yrs)	n=26	-0.099	0.632
Fasting plasma glucose ($mmol.l^{-1}$)	n=26	-0.051	0.803
HbA_{1c} (%)	n=22	0.038	0.866

Table 5.3: The correlation of TRAP with age, duration of diabetes and glycaemia in diabetic subjects.

Pearson correlation coefficients and the level of significance (p value) of the correlation within the diabetic group.

Dependant Variable	Independant Variable		B (95%CI)	p	r
a) TRAP	Age	(yrs)	1.5(-3.0,6.0)	0.519	0.030
	Duration	(yrs)	0.4(-2.0,8.2)	0.922	
	HbA_{1c}	(%)	5.8(-33.4,45.0)	0.776	
b) TRAP	Age	(yrs)	-1.9(-6.6,2.80)	0.446	0.339
	Duration	(yrs)	-2.3(-9.4,4.8)	0.532	
	Smoking		252.9(66.7,439.1)	0.018	
	HbA_{1c}	(%)	20.9(-15.4,57.2)	0.277	

Table 5.4: The correlation of age, duration of diabetes, glycaemia and smoking with TRAP in diabetic subjects using a multiple linear regression model.

Partial correlation coefficients (B), their 95% confidence interval and p values for two multiple linear regression models,a) including and b) excluding smoking as an independant variable. Diabetic subjects only. The multiple r^2 (r) value is given.

	Sub-group		TRAP	p
Gender	Male Female	(n=12) (n=14)	672.3(205.8) 652.9(148.7)	0.789
Type	IDDM NIDDM	(n=8) (n=18)	665.4(200.2) 660.2(167.7)	0.950
Complications	None Any	(n=9) (n=17)	611.8(164.2) 688.3(177.7)	0.287

Table 5.5: The comparison of TRAP values in diabetic subgroups.

The mean (SD) TRAP values in the subgroups are given. Comparisons are tested using unpaired t-tests and p values are given.

positive correlation between age and glutathione peroxidase activity was reported by Ceballos-Picot *et al* (206). Also some reports of reduced vitamin E with age (258) may be misleading in that they do not correct for lipid changes with age (259). The use of TRAP as a measure of plasma antioxidant activity is theoretically an improvement upon the use of individual antioxidants as a guide to such activity because it is contributed to by all plasma antioxidants active against peroxyl radicals and it allows for their interaction. Using individual antioxidants the situation regarding the antioxidant status in diabetes was not clear as some groups had found that some antioxidants were reduced (119-126) whilst other groups reported increases in yet other antioxidants (251). It was hoped that by using TRAP this situation might be clarified. Examining the subjects as a whole TRAP did seem to be lower in the diabetic subjects studied than in the non-diabetic subjects however when the groups were divided again on the basis of smoking it was found that TRAP was not significantly different in non-smoking, diabetic and non-diabetic individuals suggesting that diabetes alone does not have a significant influence on antioxidant status. Taken together with results of those other studies, which suggested changes in individual antioxidants, this again suggests, as with the case of ageing, that their may be homeostatic mechanisms aimed at maintaining overall antioxidant status which function effectively in diabetes. The lack of difference in TRAP in diabetic and non-diabetic non-smokers also suggests that differences, in overall plasma antioxidant status, do not contribute to the increased independent risk of vascular disease associated with diabetes or the developement of other complications and this is in keeping with the finding that TRAP in those diabetics with and without complications was not significantly different. The lack of difference between diabetics and non-diabetics is also consistent with the lack of correlation between TRAP and measures of glycaemia. Another possible explanation for the lack of difference in TRAP between diabetics and non-diabetics is that the study is insufficiently powerful to detect a difference of 10% and indeed the power to do so is only 7% - to detect a 10% difference at the 0.05 level with 80% power 64 patients in each group would have to be studied.

5.6.2. Trap And Smoking.

The finding of a difference in TRAP between diabetics and non-diabetics in the whole group but no difference in TRAP when non-smokers alone were assessed

implied that either smoking was the relevant variable or that an interaction between diabetes and smoking existed and these possibilities were tested in an analysis of variance, with diabetes and smoking as independent variables. This analysis confirmed that there was a significant interaction between diabetes and smoking in determining TRAP. This is consistent with the finding that smoking confounded the relationshp between plasma vitamin C levels and risk of angina (199) in the light of the fact that vitamin C is a contributer to TRAP (26). As a consequence of the interaction between smoking and diabetes TRAP in diabetic smokers was significantly lower than in diabetic non-smokers. Smoking may produce a lowering of antioxidant defence in a number of ways. It has often been reported that smoking is associated with reduced plasma levels of ascorbic acid (197, 199) and this may be a result of reduced intake of this vitamin by smokers (195). Why this difference in intake should occur is unknown. It may reflect the fact that smokers are in general less health conscious or more resistant to health education advice, including that regarding diet, than are non-smokers. It is also possible that smoking alters taste and dietary habits of smokers and this has been cited for the changes in intake of other nutrients such as linoleic acid in smokers (152). Smoking is related to poor social circumstances and it might have been argued that intake of nutrients in smokers could be determined by their relatively poorer income. However, in a group of pregnant women Haste *et al* has shown that income played very little part in determining micronutrient intake and that smoking habit was its most important determinant (263).

In addition to reduced intake, reduction in antioxidant levels might also occur in circumstances of increased demand and consumption, as previously discussed. Where there is doubt as to the direction of change in demand, as in the case of diabetes, interpretation of alterations in antioxidant levels becomes very difficult. Smoking is less of a problem in this respect. Cigarette smoke is both a direct source of FRs and also contains chemical species which in stimulating neutrophils results indirectly in FR production (13, 195). Harats *et al* also showed induction of lipid peroxidation acutely following consumption of 5-7 cigarettes (196). There seems little reason to doubt, therefore, that smoking is associated with increased demand for antioxidants, and thus a lower antioxidant level is consistent with increased consumption in association with smoking in diabetics. An interaction between diabetes and smoking which reduces TRAP may help to explain the even greater increased risk of vascular disease associated with smoking in diabetes compared to

that in non-diabetics. As well as indicating that greater consumption of antioxidants may occur in diabetes in conjuntion with smoking a lower TRAP, perhaps just as importantly, implies that should diabetic smokers be exposed to further sudden increase in FR dose, as is proposed after myocardial infartion or other ischaemic episode (21-23, 230, 264-266), they would be at even greater risk of FR mediated damage and it would be interesting to ascertain whether or not diabetic smokers have a particularly poor outcome following such events.

The number of smoking, non-diabetic subjects in this study is small and so I cannot reach any conclusions about the effect of smoking in non-diabetics (n=4) but it is interesting that the TRAP levels in non-diabetic subjects is not significantly different in smokers vs non-smokers. In fact, the TRAP levels in non-diabetic smokers is, if anything, higher than in the non-smokers, raising the possibility that smoking induces an adaptive overall increase in antioxidants in non-diabetics. As evidence of the possibility that adaptive responses can occur, raised erythrocyte glutathione (194) and raised alveolar macrophage ascorbate (195) have been found in smokers. Perhaps two sources of increased demand for antioxidants overwhelms the body's ability to match that demand in diabetic smokers. The effect of smoking on TRAP in diabetics and non-diabetics warrents further investigation and this study highlights the need to take into account the smoking status of subjects when investigating antioxidant levels.

The lack of correlation between HbA_{1c}, fasting plasma glucose and duration of diabetes with TRAP is not in keeping with the contention that FR activity, and so antioxidant abnormalities, in diabetes might be directly related to the degree of metabolic upset. On the other hand, HbA_{1c} is a measure of glycaemia and possibly the oxidative process involved in glycation and not of FR generation processes generally.

The lack of difference between TRAP in those with and without complications is not consistent with the hypothesis that FR activity is involved in the generation of the complications of diabetes but this study was not set up primarily to test that hypothesis and its power to detect a difference is low.

5.7. CONCLUSION

TRAP was used in this study as a measure of antioxidant status because it offered advantages over the use of individual antioxidants in assessing that status. It's use has suggested no overall change in antioxidant status in diabetes *per se* but a significant interaction between diabetes and smoking such that TRAP in diabetic smokers was significantly lower than in non-smokers. This interaction may help explain the even greater risk of smoking in diabetics. The lower TRAP in these subjects could also leave them exposed to FR damage in circumstances of increased FR load such as following ischaemic episodes. A normal TRAP in non-smoking diabetics suggests that alterations in overall plasma antioxidant status can not contribute to the increased risk of vascular disease in non-smoking diabetics. This may suggest that plasma antioxidants are not important in the processes involved in the generation of vascular disease but it remains possible that antioxidant status elsewhere such as within plasma lipoproteins or in the subendothelial space does have an important role.

CHAPTER SIX

THE RELATIONSHIP BETWEEN PLASMA ANTIOXIDANT ACTIVITY AND LIPID PEROXIDATION.

6.1. INTRODUCTION

In the previous chapter the plasma antioxidant status, TRAP,in diabetic and non-diabetic subjects was compared and some of the the factors affecting it in diabetes were explored. Antioxidants should theoretically inhibit FR processes including the oxidaton of lipid, thought to be integral to the modification of LDL to more atherogenic forms. In this chapter, using TRAP as a measure of the antioxidant status of plasma, I explore the relationship between plasma antioxidants and the ability of lipids in that plasma to oxidise, that is their oxidisability. The reactions involved in lipid oxidation have been discribed in chapter three. The method used in this chapter to assess FR lipid oxidisability is a variation on standard methods Here I will first discuss the possible pathological relevance of lipid oxidation and then describe the method employed to assess lipid oxidation and discuss the rational for it's use.

6.1.1. Relevance Of Lipid Peroxidation To Atherosclerosis.

Since its identification, lipid oxidation has been implicated in the pathogenesis of many disease processes. In the context of atheroma it is of interest in several ways. Lipid oxidation products are markers of FR activity generally and of attack on lipids more specifically. Apart from FR markers, they may have a pathological significance of their own. As already discussed, in the generation of a more atherogenic LDL particle, it is believed that the aldehyde products of lipid peroxidation react with and modify ApoB hence altering LDL receptor binding and uptake by macrophages resulting in foam cell formation (48). Lipid peroxides may also have atherogenic activity distinct from any connection with LDL. Lipid peroxides within erythrocyte membranes may alter haemorheological parameters favouring thrombosis (68), also, they have been shown to inhibit antithrombin III activity (267). In addition, platelet aggregation and endothelial cell prostacyclin production have been shown capable of modulation by lipid peroxides (268) and

direct endothelial cell injury and enhanced permeability have been induced by lipid peroxides (269, 270). All of these features of lipid peroxides suggest potential mechanisms whereby they may contribute to atherosclerosis.

Lipid peroxidation may also have a role in other disease mechanisms but it needs to be acknowledged, as pointed out by Dormandy (218), that it is integral to in many physiological processes and not always a harmful phenomenon.

6.1.2. Lipid Peroxidation In Diabetes And Atherosclerosis.

There is evidence that more lipid peroxidation does occur in diabetes. There is some debate, however, as to whether or not this is only true in the presence of diabetic complications. Compared to controls, erythrocyte membranes from diabetic subjects were found to have more TBARS and the concentration of these substances correlated significantly with markers of glycaemic control (68). Plasma lipid peroxides were found to be higher in diabetic subjects compared to controls and higher still in those diabetic subjects with "angiopathy" (microvascular disease) (210). However, Sato *et al* reported significantly higher plasma lipid peroxides in diabetics compared to controls but when the diabetics were divided into those with and without angiopathy only those with angiopathy were significantly different from controls (65). Similarly Jennings *et al* found increased diene conjugates only in those with microangiopathy (70). Increased lipid peroxides were also reported in Type 2 diabetes by Velázquez *et al* but only in those with macrovascular disease and non-diabetic individuals with macrovascular disease similarly had elevated TBARS when compared with controls (67). This confirmed an earlier study which also showed that atherosclerosis was associated with increased levels of TBARS but that diabetes had no independent effect (271).

6.1.3. Measurement Of Lipid Peroxidation.

Many methods have been developed for the measurement of FR lipid oxidation (32). Those most widely used are based on assessing the concentrations of the products of the reactions outlined in chapter three (Fig. 3.1.). As discussed in section 3.1.1., because most of the measurable products of oxidation are actually derived more specifically by peroxidation, peroxidation is the term commonly used when describing lipid oxidation and I use it in this chapter. Total diene conjugated fatty

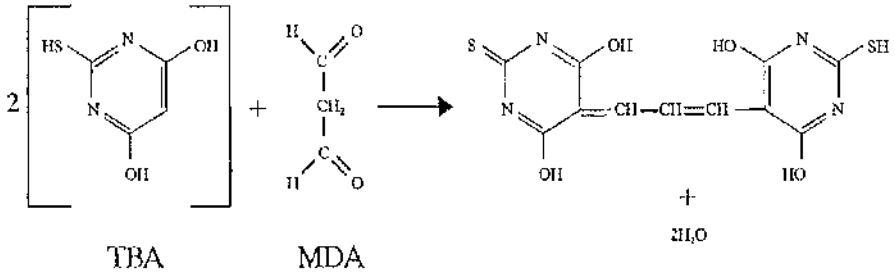
acids (tDC) and thiobarbituic acid reactive substances (TBARS) were measured in this study in the assessment of lipid peroxidation.

a. Total Diene Conjugation (tDC)

The products of lipid peroxidation which have a diene conjugated system of double bonds are typically detected by U.V. absorption spectroscopy, made possible by the fact that such systems of double bonds have a characteristic absorption peak at 230-240nm. If all of the lipids in a sample are extracted into solvent the concentration of total diene conjugates (tDC) within the sample can measured spectrophotometrically with the results expressed in arbitrary OD units. Such a measurment was used in this study as opposed to the measure of the absolute concentration of specific diene conjugated fatty acids using HPLC and UV detection (DCFA) described and employed in chapter three.

b.Thiobarbituric Acid Reactive Substances (TBARS)

Malondialdehyde (MDA) and hydroperoxides are formed during lipid peroxidation and MDA and those hydroperoxides which can be broken down to fragments including MDA, can be measured by their ability to react with thiobarbituric acid (TBA).



TBA MDA

Reaction of MDA with TBA gives a pink complex which absorbs at 532nm and fluoresces at 553nm. The test for thiobarbituric acid reactive substances (TBARS) is one on the most frequently used in assessing peroxidation but its interpretation may cause confusion if it is not realised what it is actually measuring. Hydroperoxides are not measured directly but are broken down to MDA during the test and measured in terms of MDA equivalents. The standard used is MDA and

the results are expressed in terms of μmol.MDA.l^{-1}. Hence, the test directly measures all of the MDA present in a sample and, indirectly only those hydroperoxides which breakdown to MDA. However, there is very little MDA present in fresh biological samples as it is believed to be removed rapidly *in vivo* (65, 272) and so most TBARS derive from hydroperoxides.

Despite their common use, there are well documented problems with these methods of measuring tDC and TBARS in that they are non-specific (32, 272). Moreover, sources of tDC and TBARS other than FR attack on lipids have been identified. In chapter three a study was described which suggested that diet is a source of DC species. Hydroperoxides are also the physiological intermediates of enzymic reactions, such as those involved in eicosanoid metabolism (270, 16) and may also be ingested.

6.1.4. Principle Of The Peroxidisability Assay.

The factors which determine the extent to which the lipids in plasma peroxidise, which I will refer to as their peroxadisability, are not well understood but it might be anticipated that the nature of the lipids, as substrate, and a balance between plasma oxidants and antioxidants would be involved. I wished to assess the importance of antioxidants in this context by looking at the relationship between plasma antioxidants (TRAP) and plasma lipid peroxidisability. Since peroxidised material is almost certainly rapidly removed *in vivo* (27), and there may be several sources of such material in plasma, it was felt that a single measurement of the concentration of peroxidised materials in plasma would reflect a balance between production and removal and not necessarily peroxidisability and that peroxidisability could better be assessed by measuring the rise in peroxidation products in isolated plasma over a period of time. The lipids in plasma will peroxidise spontaneously but, if stressed, the process can be accelerated. In this study plasma lipids were "stressed" by maintaining plasma at 37°C and pH 5.5 for 24 hours (273) and peroxidisability was assessed by measuring the changes in absorbence due to tDC, and in concentration of TBARS. The relative volume of serum to acetate buffer required to acheive these final conditions was established and reported by McFarlane (273). By measuring the change in these species in these circumstances *in vitro* the possibility of them arising from alternative sources, such as diet and cellular enzymatic processes, is also removed.

6.1.5. Summary.

Lipid peroxidation initiates the process of LDL oxidation which enhances its atherogenicity. Products of lipid peroxidation also have other potentially atherogenic effects. The mechanism of lipid peroxidation involves FR activity and should by impeded by antioxidants. Many groups have shown that lipid peroxidation products are more abundant in diabetes. Others have shown reduced antioxidant levels in diabetes. I wished to determine if the extent to which lipids within plasma could be oxidised was influenced by the antioxidant activity of that plasma. Since the concentration of lipid peroxidation products in plasma may not reflect the degree to which peroxidation occurs, because these species are both removed from plasma and arise there by other mechanisms, thus an *in vitro* assay of the extent of peroxidation in a set period of time was used to reflect "peroxidisability" and the relationship between "peroxidisability" and antioxidant activity (TRAP) was studied.

6.2 HYPOTHESIS.

The extent to which the lipids in plasma from diabetic subjects peroxidise will be inversely related to plasma antioxidant activity, as measured by TRAP.

6.3. SUBJECTS AND STUDY DESIGN.

Peroxidisability was measured as the second limb of the study described in chapter five and the subjects in which it was measured were those diabetic subjects described in chapter five.

6.4. METHODS

The analytical and statistical methods used were described in chapter two.

6.4.1. Assays performed

These are listed in section 5.4.1.

6.5. RESULTS.

6.5.1. Peroxidisability And TRAP.

There was no correlation between TRAP and either plasma peroxidisability as measured by the change in total diene conjugated species (tDC) ($r=0.008$, $p=0.972$) (Fig 6.1), or as measured by change in thiobarbituric acid reactive substances (TBARS) ($r_s=0.184$, $p=0.390$) (Fig 6.2) in diabetic subjects. The two measures of peroxidisability did not significantly correlate with each other ($r_s=0.100$, $p=0.615$) (Fig 6.3).

Change in tDC significantly correlated with total cholesterol concentration ($r=0.575$, $p=0.001$) (Fig 6.4) but not with total triglyceride concentration ($r=0.105$, $p=0.605$), change in TBARS did not correlate with either total cholesterol ($r_s=0.104$, $p=0.597$) or triglyceride concentration ($r_s=0.202$, $p=0.302$).

Neither measure of peroxidisability was significantly different in smokers compared to non-smokers (Smokers vs non-smokers :- change in tDC: 1.32(0.67) vs 1.40(0.65) OD units, $p=0.752$); change in TBARS: 4.69(3.27,8.62) vs 6.89(4.69,9.24) μmol MDA.l^{-1}, $p<0.100$).

6.5.2. Peroxidisability And Diabetes.

Neither measure of peroxidisability correlated with plasma glucose, HbA_{1c}, duration of diabetes or age (Table 6.1). Comparing Type 1 and 2 diabetic subjects there was no significant difference in the change in tDC (1.20(0.51), vs 1.42(0.69) OD units, $p=0.393$) or in TBARS (7.42(5.02,13.29) vs 6.28(3.57,7.75) μmol MDA.l^{-1}, $p = 0.231$).

Comparing subjects with no complications with those having any form of complication or with only macrovascular complications also revealed no significant difference in either measure of peroxidisability (Table 6.2).

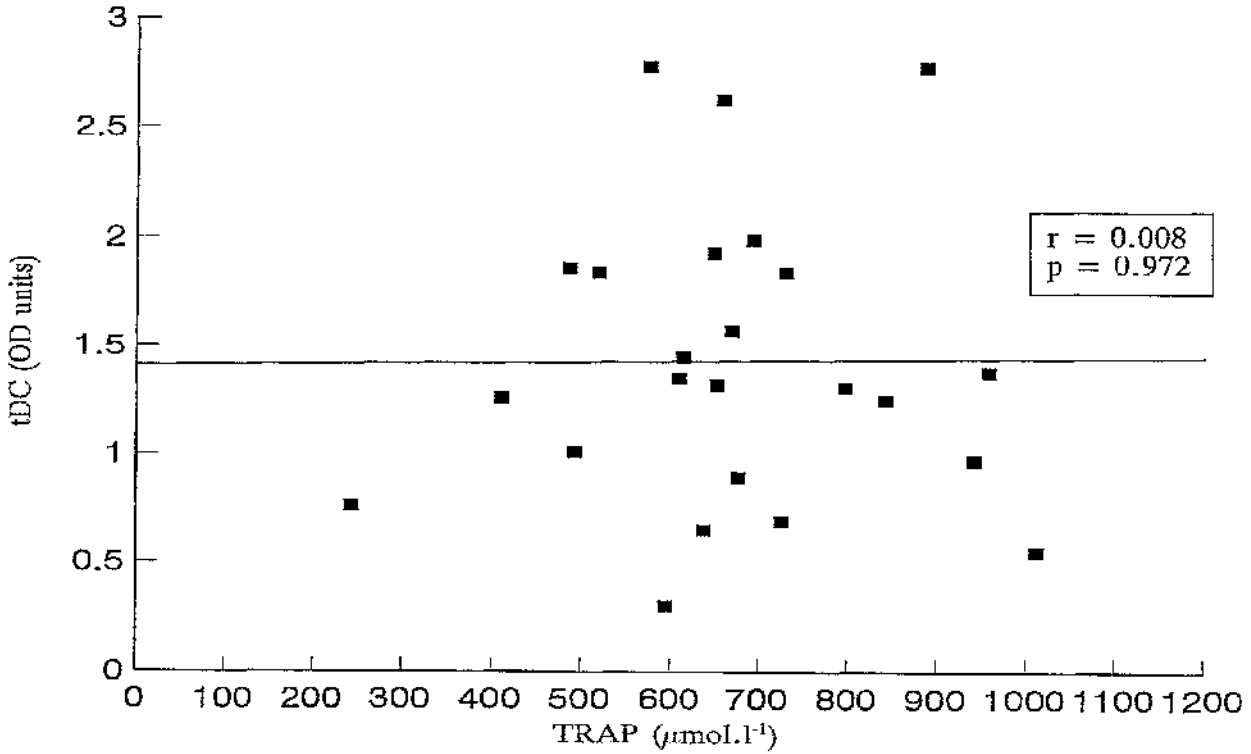


Figure 6.1: The correlation between plasma peroxidisability, as measured by change in diene conjugated fatty acids (tDC),and TRAP.

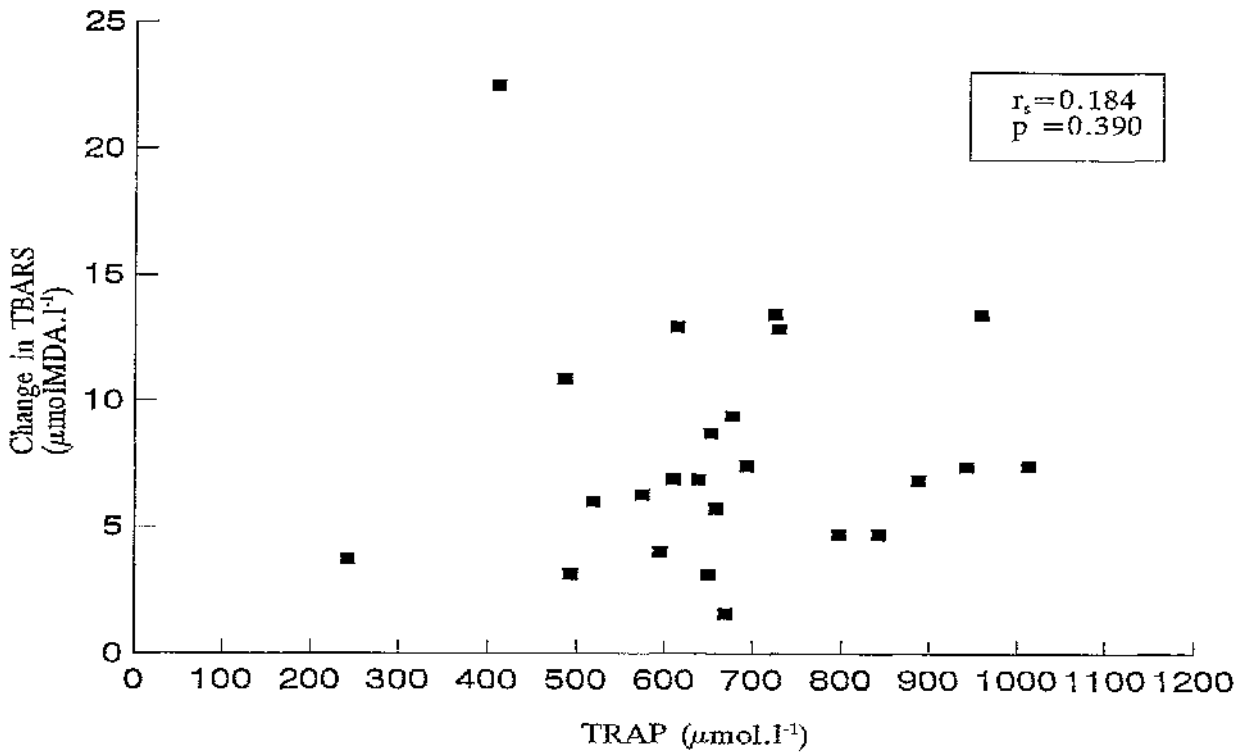


Figure 6.2: The correlation between plasma peroxidisability, as measured by change in thiobarbituric reactive substances (TBARS), and TRAP.

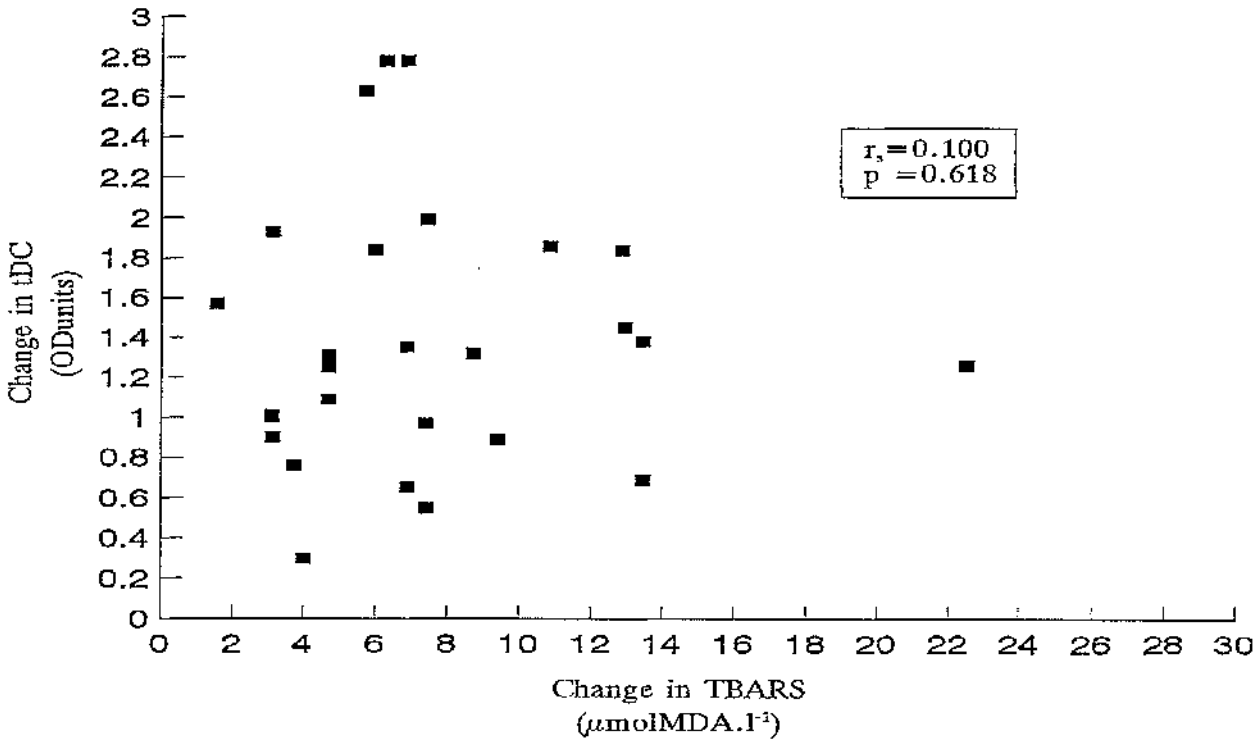


Figure 6.3: The correlation between two measures of peroxidisability, that is between the change in total diene conjugated fatty acids (tDC) and the change in thiobarbituric acid reactive substances (TBARS).

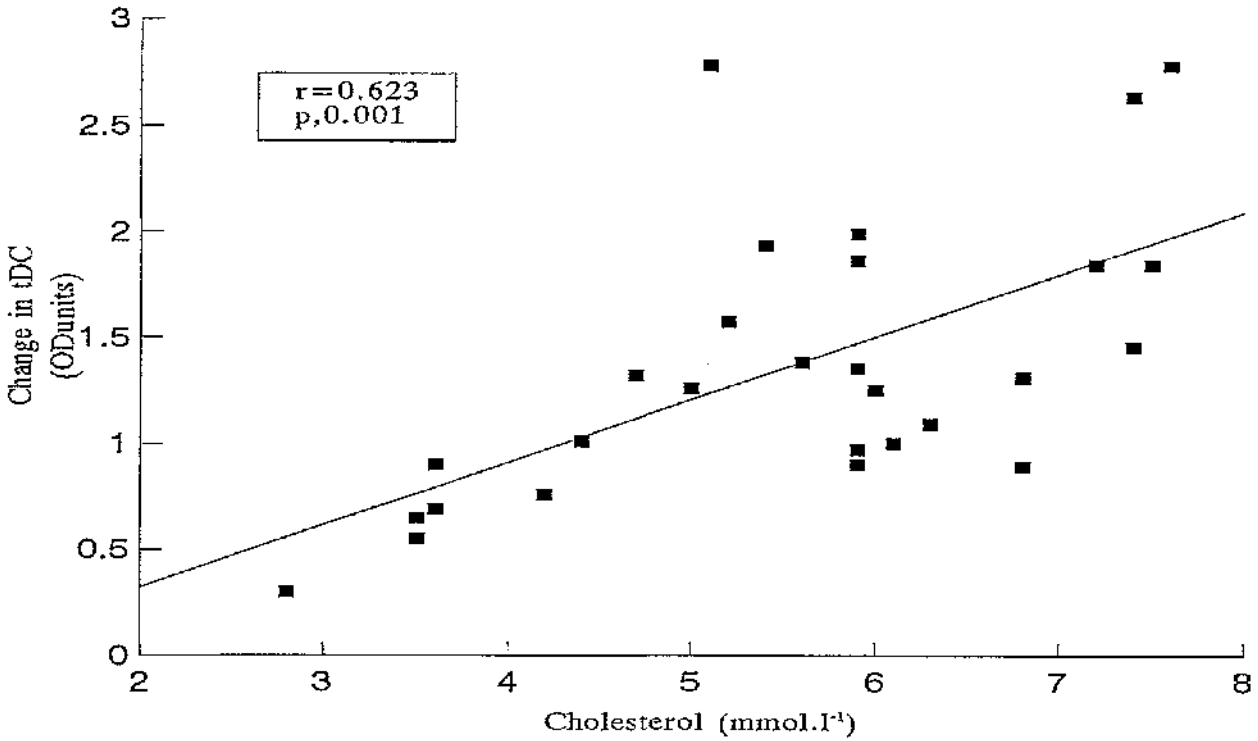


Figure 6.4: The correlation between plasma peroxidisability, as measured by the change in diene conjugated fatty acids (tDC), and total cholesterol.

Independent Variable	Peroxidisability			
	Change in tDC (OD units)		Change in TBARS (μmol.l^{-1})	
	r	p	r_s	p
Fasting plasma glucose (mmol.l^{-1})	-0.103 n=28	0.601	0.306 n=28	0.133
HbA_{1c} (%) (NR=6.5-8.5)	-0.240 n=23	0.260	-0.250 n=23	0.250
Duration (yrs)	0.016 n=28	0.937	0.067 n=28	0.736
Age (yrs)	0.041 n=28	0.835	0.093 n=28	0.640

Table 6.1: The correlation of peroxidisability of plasma lipids with glycaemia, duration of diabetes and age.

Peroxidisability was assessed by the change in total diene conjugates (tDC) and by the change in thiobarbituric acid reactive substances (TBARS). Spearman rank correlation coefficients (r_s) of Pearson coefficients (r) and the level of significance of the correlation (p values) are given.

Complications	Change in tDC (OD units)		Change in TBARS (μmol.l^{-1})	
None (n=12)	1.58(0.69)		5.20(3.41,8.41)	
Any (n=16)	1.20(0.59)	p=0.142	6.89(4.35,10.82)	p>0.1
Macrovascular (n=3)	1.24(0.48)	p=0.369	12.94(4.4,13.3)	p>0.1

Table 6.2: Comparison of peroxidisability in those with and without complications.

Values are mean (SD) or median (25th, 75th percentile). Comparisons between groups are tested using unpaired t-tests or Mann-Whitney U-test and pvalues refer to the comparison with the group having no complications.

6.6 DISCUSSION

6.6.1 The Relationship Between Antioxidants And Peroxidisability

It has been reported that diabetic lipids, both plasma and membrane bound, are more oxidised than those of non-diabetics (65-70, 218). They may be more oxidised because of reduced availability of plasma antioxidants, although I did not find a reduction in total plasma antioxidants (TRAP) in diabetes other than in association with smoking as discussed in the previous chapter. In this study, using an *in vitro* assay, the peroxidisability of lipids in plasma from diabetic subjects was determined, and the correlation between peroxidisability and TRAP levels examined. No correlation was seen, hence the hypothesis that peroxidisability of diabetic lipids is inversely related to plasma antioxidants (TRAP) is not supported by the results of this study.

Several reasons may exist for this lack of association. The *in vitro* conditions by which peroxidation was stimulated were arguably unphysiological and so the test may be an unrepresentative model of *in vivo* peroxidation. However, the fact that products arise that are known to occur *in vivo* suggests it is not completely unrepresentative and, in conditions of tissue ischaemia, a tissue pH of 5.5 could be achieved *in vivo*. Also, others have employed similarly "unphysiological" approaches to stress lipids *in vitro*, both isolated and in plasma, whilst studying the process of peroxidation (27, 47, 274). Based on comparable reasoning to this study, Arshad *et al* conducted a study in which Cu^{2+} and H_2O_2, in various concentrations and combinations were used in order to stress plasma and they measured the rise in concentration of TBARS and cholesterol oxides after a 2hr period of incubation. They then took this rise as an indicator of the susceptability of plasma lipids to peroxidation. They compared diabetic and non-diabetic plasma and concluded the former was more susceptible to peroxidation and that the balance between substrate factors, oxidants and antioxidants favoured oxidation in diabetes. I did not compare diabetics and non-diabetics but tried to verify the hypothesis that antioxidants did influence such measurments of lipid peroxidation. Even though I used TRAP, with it's advantages as a measure of antioxidant status, I did not find confirmation of the hypothesis.

Assuming that the measurement of lipid peroxidisability used is valid then other explanations for the lack of correlation between TRAP and peroxidisability might lie

in the complexity of the oxidation process, partly introduced by the parcelling of plasma lipids into lipoprotein particles. Parcelled towards the core of the particle, in a hydrophobic environment, lipids are relatively removed from what happens in the aqueous phase of plasma. This, and other complexities, are discussed in the following sections.

a. Antioxidants and the stage of peroxidation

Frei *et al* studied the time course of peroxidation of lipids in plasma and noted that the endogenous lipids are oxidised in a similar pattern to the exogenously added linoleic acid in the TRAP experiment (Fig 5.4) (27). A lag period existed between exposure to FR and the initiation of peroxidation, the lag period was presumed to be a reflection of endogenous antioxidants in plasma. The lag period was then followed by a period of peroxidation. The lag period coincided with the disappearance of ascorbic acid (AA), peroxidation only beginning when the AA had been consumed. From this it was concluded that the water soluble AA was a major protector of the lipids in plasma. This conclusion may seem at odds with the result of the present study in which TRAP, to which AA contributes, did not relate to peroxidisability. However, the experimental design here is such that the aspect of peroxidation observed is not the lag but the later peroxidation phase, and specifically the extent to which peroxidation occurs during the incubation period. Frei does not comment on how this aspect of the peroxidation relates to antioxidants. If AA is consumed before this stage is entered, AA could not be expected to influence it. The current study suggests that the other contributors to TRAP do not either. AA has been shown to contribute up to 24%, and vitamin E 5-10%, of the TRAP activity whilst uric acid and plasma proteins contribute much of the rest (26, 260). The results of studies such as that of Babiy *et al* are in keeping with the lack of influence of other antioxidants, specifically vitamin E, on the extent of lipid peroxidation (144). Although looking at isolated LDL as opposed to plasma, and measuring vitamin E as opposed to TRAP, they measured the rise in lipid peroxides in response to a dose of γ irradiation, as oxidant stress, and found no correlation between vitamin E content of LDL and this rise, which they termed "LDL oxidisability" (144). Hence, overall it would seem that the extent of lipid peroxidation may not be influenced to any detectable extent by antioxidants.

It is possible that unidentified antioxidants or the nature of the PUFA, as oxidisable substrate, may be a more important determinant of peroxidisability, as it is measured

in this study than are the contributors to TRAP. I did not assess the concentration of individual FA, but it is worth noting that peroxidisability, as measured by change in diene conjugates, although not change in TBARS, was significantly related to the concentration of total plasma cholesterol but not triglycerides. This may be a statistical error but it might reflect the fact that the cholesterol rich lipoprotein, LDL, is more easily oxidised than the TG rich particles. It might also reflect the fact that although cholesterol is not as readily oxidised as PUFA, LDL contains cholesterol which has been esterified predominantly to linoleic acid. Linoleic acid has been shown to be the major source of lipid peroxidation products during LDL autoxidation *in vitro* (142). Lack of correlation between the measures of peroxidisability and total plasma TGs might be explained by the fact that a relatively smaller proportion of the FAs esterified within TGs are polyunsaturated and so oxidisable. However there are influences upon the correlations between measures of peroxidisability and, TG and cholesterol, other than the relative oxidisability of PUFA within cholesterol esters and triglycerides. These same influences may explain why anomalies arose such as the finding that tDC and TBARS did not correlate with each other and the finding that whilst the change in tDC correlated with cholesterol, the change in TBARS did not. These are discussed further in section 6.6.1c.

Even if antioxidants do not have a major role in determining the extent of lipid peroxidation once started they may affect other important aspects of peroxidation. Considering again the model in which lipids resist oxidation until ascorbic acid, in the case of plasma (27), and vitamin E in the case of isolated LDL (28, 46, 213), are consumed, it was proposed that the time for which lipids could resist oxidation, the lag period, would be determined by antioxidant concentration. However, even that proposal may be too simplistic as it has been shown in several studies that the lag time does not correlate with antioxidant concentration (27, 28, 46). Nevertheless, in a study on isolated LDL undergoing copper stimulated oxidation, although the lag time did not correlate with the vitamin E content of LDL, it was noted that increasing the vitamin E content of any individual's LDL did increase the lag phase (46). So, assuming when looking at an individual that other variables are held constant, this suggests that antioxidants do have a role in determining lag but that again other factors are involved and even in the lag phase substrate features may be important.

b. Antioxidant solubility

Work such as that of Doba *et al* suggests that the phase, lipid *vs* aqueous, in which the FR initiator and antioxidant exist may also help determine the effectiveness of their antagonism (275). In agreement with Frei *et al* (27) they showed that water soluble ascorbic acid (AA) effectively induced a lag in peroxidation of phospholipid liposomes when the water soluble initiator ABAP was used but did so only poorly when using the lipid soluble initiator, DMVN (azo-bis(2,4-dimethylvaleronitrile)) (275). In the TRAP assay the initiator is the water soluble ABAP and so TRAP is mainly determined by antioxidants within the aqueous phase and how the antioxidant system interacts to control aqueous phase radicals. The site of the initiator in the conditions employed to produce peroxidation in this study is not known but may be the lipid phase. It is possible, as argued by Halliwell, that lipid hydroperoxides are present in trace amounts within the lipoproteins and in the presence of transition metals, they decompose with the formation of peroxyl and alkoxyl radicals (221). Being in the lipid phase, these radicals may never be available to water soluble plasma antioxidants explaining the lack of correlation between TRAP and peroxidisability. The corollary might then be that there should be an inverse correlation between peroxidisability and lipid soluble antioxidants. Transition metal ions were not added in the peroxidisability experiments performed but are almost certainly present in trace amounts in the buffer. Also, at pH5.5 the binding of these ions within low molecular weight, and protein, complexes in plasma may well be reduced, as has been shown for the binding of Fe^{2+} within transferrin (247). The possibility that the metal catalysed process is initiated in the lipid phase is supported by studies in methyl linoleate micelles. When the micelle surface was positively charged and so repelling positively charged ions, oxidation was inhibited (30, 276). Also, if the micelles were treated with triphenylphosphate to remove traces of hydroperoxides, again metal catalysed oxidation did not occur (30, 276).

If hydroperoxides do exist in trace amounts in lipoproteins, the question of their source arises. Diet is a possibility. Lipoxygenase enzyme activity also results in hydroperoxide production. The transfer of these products to LDL has been proposed as an alternative way to initiate LDL oxidation (49).

c. The specificity of the antioxidant

Frei's work points to yet other complexities in the peroxidation of lipids. Even when employing two initiators both releasing FR into the aqueous phase the pattern

of antioxidant consumption differed. The two initiators, ABAP and activated polymorphonuclear leucocytes, would have released different radicals. Their results suggest that the antioxidant systems operate differently, depending upon the specific radical involved; different radicals first encounter the system at different points and may experience a different antioxidant capability.

Taken together, all of these studies imply a complex model of how FRs and antioxidants interact in the process of lipoprotein peroxidation. It may be unwisely crude to generalise that antioxidants inhibit FR oxidation of lipoproteins. The nature of the radical, its solubility and the situation in which it is generated will affect which antioxidant best scavenges it. The substrate will also influence the outcome. Failure to take all of these factors and their interactions into account may help to explain why correlations between antioxidants and peroxidisability have been so difficult to establish (27, 28, 46, 144). Returning to the present study, perhaps a correlation between TRAP and peroxidisability might have been seen had a different oxidation stress been applied to the plasma, as peroxidation might then have been antagonised by the antioxidants contributing to TRAP. These arguments have experimental and clinical implications. Experimentally, great care will be needed in the choice of 'models of stress' when comparing studies, and when making inferences regarding *in vivo* processes from *in vitro* studies. Clinically, the implication is that if different disease processes produce oxidative stress in a different way the prophylactic or therapeutic response will also have to be tailored accordingly.

Before concluding I would like to come back to the validity of the peroxidisability assay. The two variables used in this study to assess peroxidation, the change in tDC and TBARS, do not correlate with each other suggesting that they may not equally well represent peroxidation. Which is the more reliable? As discussed by Halliwell (13), no currently available test of peroxidation is entirely satisfactory. They all measure different things and the most appropriate must be judged by a knowledge of the circumstances. There are several reasons why in the circumstances here that changes in TBARS may be a less reliable index of peroxidation than total DCs:-

i) Even though I was looking at the change in these species, since plasma was used the lower specificity of the TBARs assay may remain a problem.

ii) Esterbauer, looking at copper stimulated oxidation of isolated LDL, saw good correlation between the rise in tDCs and TBARS once oxidation had begun but only

up to the point of maximum DC formation (213). With longer incubation, as in this study, the correlation was lost. The loss of correlation may be because hydroperoxides are less stable than non-peroxide DCs. This means that a proportion of both species formed will decompose during the incubation period, so that the concentrations measured at the end of the incubation will be an underestimate of the total formed but the error will be most marked for the hydroperoxides.

In addition to helping to explain the lack of correlation between the measured change in TBARs and tDC during the incubation period, it decomposition of products does occur it could also help explain the apparent lack of correlation between measures of peroxidisability and TRAP. If decomposition of products is continuously occurring then measurement of peroxidisability could be sufficiently inaccurate to mask any correlations.

Another concern needs to be addressed. If the time course of peroxidation is such that it is complete before the second measurement of products is made the assay would actually be measuring maximal extent of peroxidation and not peroxidisability. The time course of peroxidation was not examined in this study. It needs to be verified before further work using this method of measuring peroxidisability is undertaken. All other methods of measuring peroxidisability need to clarify that the product being measured is stable under the conditions of the experiment and that a plateau phase has not been reached at the time of repeat measurements.

iii) MDA, and so TBA reactivity, can be generated only by PUFA with 3 or more double bonds (142). Hence, linoleic acid hydroperoxides with two double bonds, despite being the major product of oxidation and the most stable of the hydroperoxides formed, will not be detected by the TBARs assay. They will, however, contribute to the signal representing DCs. Overall, in the circumstances of this study DC measurements are felt likely to be the more appropriate measure of peroxidation. These points may help to explain how the anomoly mentioned in section 6.6.1a could arise whereby the change in total DC correlated with cholesterol but the change in TBARS did not. However these considerations also suggest that peroxidisability, as measured, may be so influenced by the conditions of the test and reflect chemistry other than the relative oxidisability of PUFA esterified to cholesterol or glycerol that caution is required in interpreting the results of this study and the results of other studies in which similar techniques might be used.

Another difficulty in this area is that it cannot be assumed that equal amounts of oxidising FAs will go to form non-peroxide DC and TBARS, and the factors which

control the balance of oxidation pathways taken are unknown.

6.6.2. Other Influences On Peroxidisability And Its Relevance.

Plasma peroxidisability did not relate to plasma glucose concentration. This is in keeping with the finding that addition of glucose to human plasma did not increase lipid peroxide formation *in vitro* (274) but appears to contradict the finding that human isolated LDL did show enhanced peroxidation when incubated with glucose (102) albeit at supraphysiological concentrations (200mM). It may be that in plasma, and so possibly *in vivo*, there are so many factors which influence peroxidisability that the enhancing effect of glucose on LDL peroxidation is swamped. Taken together with the finding of others that nevertheless, there may be more peroxidised lipids in plasma and tissues of diabetics (68-73, 210), this suggests that diabetes may have effects on lipid oxidation, not only at the level of the glucose directly increasing peroxidation or altering the lipids such that they subsequently oxidise more easily, but by altering the oxidative stresses, or by reducing the clearance of oxidised lipids or by decreasing antioxidants.

My own study and others, including those discussed here, attempt to help define what determines variability in lipid peroxidation. However, in suggesting the complexity of *in vivo* peroxidation they raise another question, namely if there are different phases of lipid oxidation is one pathophysiologically more relevant than another? Very little work has been done on this but recently the duration of the lag phase, during oxidation of LDL isolated from survivors of myocardial infarction, was shown to be inversely related to the severity of coronary atherosclerosis seen at angiography (140). In keeping with the concept that the nature of the substrate might be as important as the concentration of antioxidant, it was also found that the duration of the lag phase was inversely related to LDL triglyceride content (140). The pathological relevance and correlates of the peroxidation phase as opposed to the lag phase have not been similarly studied. I did not set out to explore its relevance and my groups are heterogeneous and small, however, peroxidisability was not significantly different in those with and without complications. If the development of the complications of diabetes is related to FR activity, including lipid peroxidation, this suggests that "peroxidisability", as measured in this study, may not be a relevant feature of peroxidation.

There was no significant difference between subjects with Type 1 and Type 2

diabetes in terms of peroxidisability. If peroxidisability is shown to relate to the development of atherosclerosis, its similarity in Type 1 and 2 diabetics would be in keeping with the excess risk of this complication in both types of diabetes. Smoking was not seen to influence peroxidisability. It has also been shown not to alter the duration of the lag phase of isolated LDL oxidation (140).

6.7. CONCLUSION

Support for the hypothesis that the peroxidisability of plasma would be inversely related to plasma antioxidants (TRAP) has not been found in this study. It is possible that the physical parcelling, and so relative isolation, of lipids within lipoproteins means that oxidative stresses of different types might be antagonised by different combinations of antioxidants. Perhaps had another oxidant stress been applied in this study a relationship between peroxidation and TRAP would have been observed. It may also be that the presence of lipids within lipoproteins means that lipid soluble antioxidants will be more important determinants of peroxidisability than TRAP which is mainly contributed to by water soluble antioxidants. The nature of the substrate may also influence peroxidisability.

Peroxidisability was not related to glycaemic control and so enhanced peroxidisability would not offer any explanation for the association of level of glycaemia with the developement of diabetic complications and consistently no difference in peroxidisability between those with and without complications was found. The lack of correlation between glycaemia and peroxidisability also suggests that a raised glucose concentration *per se* is not enough to enhance lipid peroxidation detectably.

The methods availible for the study of peroxidation are frought with difficulties at all levels. Although the method used here offered some advantages, several anomalies were foun which raised doubts about just how valid a measure of peroxidisability it was. These doubts need to be born in mind when considering the interpretations here.

CHAPTER SEVEN

VITAMIN E STATUS IN DIABETES.

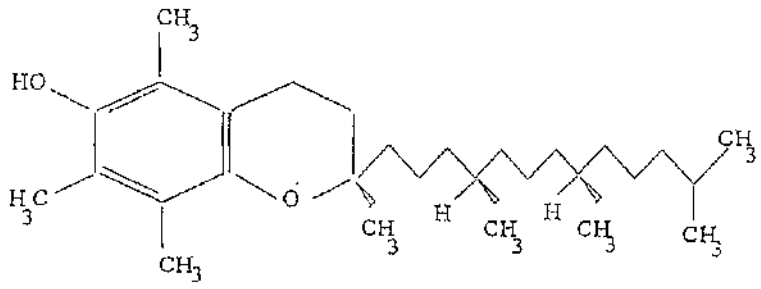
7.1 INTRODUCTION

In the previous study the total peroxyl radical antioxidant activity of plasma (TRAP) in diabetic subjects was measured. No relationship between TRAP and the peroxidisability of plasma lipids was observed. Several possible reasons for this were discussed. One possibility is that lipid soluble antioxidants are the more important protector of lipids, and are poorly represented by TRAP. Therefore, in this study, attention is turned to vitamin E, the major lipid soluble antioxidant. The need to measure vitamin E status as opposed to simply vitamin E concentrations is explained and vitamin E status in diabetes is then compared with that in non-diabetics. Also, the relationship between vitamin E status and lipid peroxidation, as assessed by plasma TBARS, is explored.

7.1.1. Vitamin E As An Antioxidant

a.The Role Of Vitamin E

The tocopherols and tocotrienols are a group of structurally similar molecules capable of antioxidant activity. Vitamin E is a generic term applied to a group of these molecules which have the same biological activity as the most common and reference compound α-tocopherol.



α-TOCOPHEROL

Vitamin E, which can react with and so inactivate, several oxidising FRs, such as $O_2^{\cdot-}$, $OH^{\cdot}$, peroxyl and alkoxyl radicals, functions as a chain breaking antioxidant

(219). The α-tocopherol radical which results is stable and not reactive enough to propagate a chain reaction and, as discussed earlier, can be reactivated by interaction with vitamin C (262, 277). The hydrophobic nature of vitamin E localises it, *in vivo*, to lipid-rich sites such as membranes and lipoproteins. Within these sites α-tocopherol molecules are believed to be aligned in such a way that the non-polar tail is deep within the hydrophobic core of the structure and the polar, reactive, alcohol group towards the surface (219). However it is mobile within these structures, moving both in parallel and perpendicular to the surface (219). This ability to move, and its links with surface and core, may explain how vitamin E can both interrupt FR reactions at the core, yet be reactivated by the strictly aqueous phase vitamin C.

In plasma, vitamin E is present in all classes of lipoproteins, including LDL (278). Within LDL, α-tocopherol is the major antioxidant so far identified but γ-tocopherol, β-carotene, retinyl stearate and lycopine also occur (28). Evidence that vitamin E functions as an antioxidant comes from kinetic studies of lipid peroxidation *in vitro*. During these kinetic studies, oxidation of isolated LDL in buffer has been followed by the appearance of DCFA, TBARS or a modified LDL taken up more rapidly by macrophages (28,46). Oxidation was seen to occur only after a time lag, which coincided with the loss of antioxidants, principally α-tocopherol. The duration of the lag period varies widely from individual to individual (213), as does the vitamin E content of LDLs and although this raised the possibility that the lag might be due to vitamin E, its duration has not been shown to correlate with the vitamin E content of LDL, suggesting that vitamin E is not the sole determinant of the lag period (46). Reasons similar to those discussed in chapter six to explain lack of correlation between TRAP and oxidation of plasma lipids could be applied again here as could the argument that the nature of the substrate also influences the lag period. Despite these considerations, the lag period can be used as an estimate of LDL's resistance to oxidation.

The importance of vitamin E to the protection of LDL is further suggested by the finding that enriching LDL with α-tocopherol, by adding it to the medium or by supplementing the diet, prolongs the lag period (28, 46). Although vitamin E is an important antioxidant, *in vivo* its function may be greatly affected by its interaction with vitamin C.

b. The Interaction Between Vitamin C And Vitamin E

Vitamin C, a water soluble antioxidant, can also prolong the lag phase when added to isolated preparations of LDL (213, 255) and may do so in two ways. In addition to recycling the α-tocopherol radical (262, 277), it may also intercept FR in the aqueous phase, hence preserving the α-tocopherol within lipoproteins (275, 255). These interactions may be important *in vivo*, where LDL circulates in plasma in which vitamin C is dissolved. In fact, in plasma, there is a suggestion that vitamin C is the major antioxidant protecting (27) plasma lipids as they appeared to start to peroxidise when vitamin C was consumed and suggesting that vitamin E has little role in that setting. However, the relative importance of vitamin C and vitamin E may depend upon the circumstances (275, 276) and the following model suggested by the work of Doba *et al* may explain why the importance of vitamin E may be obscured.

During attack by a FR generated in, or released into, the aqueous compartment vitamin C initially interrupts the majority of radicals, such that they never reach the lipoprotein. Any that do get through this barrier preferentially react with vitamin E, producing a vitamin E· radical which is reactivated by vitamin C. During this period, vitamin C concentration falls and vitamin E is preserved. Eventually vitamin C concentration falls to a level at which it can no longer maintain the equilibrium and vitamin E consumption ensues whilst vitamin C consumption continues. Lipid peroxidation begins when their concentrations are no longer sufficient to prevent FR attack on the lipids.

Should the attacking FR be generated in the lipid component, vitamin E is primarily attacked but is reactivated by vitamin C. Vitamin C levels fall whilst vitamin E is again preserved and apparently not involved. Eventually, vitamin C concentration falls to a level which cannot sustain vitamin E, vitamin E levels decline and lipid peroxidation begins.

In such a model, although vitamin C does protect against FRs arising in the lipid compartment it can do so only if vitamin E is also present and the effect of the two together is then greater than the simple sum of their effect if alone (275). Hence, although vitamin E is an important antioxidant, it may be that in the more complex situations which might arise *in vivo* these interactions with vitamin C will greatly influence its effectiveness. *In vivo* this interaction is even more useful as vitamin C can be recycled. However, there are situations where the interaction can fail. Frei *et al* noted that when the FR source was activated polymorphonuclear

leucocytes (PMN), Vitamin C was consumed but lipid peroxidation occurred despite levels of vitamin E being maintained (27). This could be accounted for by the generation of a radical capable of reacting with vitamin C and lipids but not vitamin E.

7.1.2. The Role Of Vitamin E In Protection From Atherosclerosis.

Epidemiological evidence exists that plasma vitamin E concentrations are inversely related to mortality from ischaemic heart disease (198, 279) and risk of angina (199). Apart from its possible role in limiting LDL oxidation other actions of vitamin E make it of interest in the study of atherosclerosis in diabetes. As well as lipoproteins, lipids within membranes are also vulnerable to attack. Jain *et al* have shown that erythrocyte membranes in diabetes are indeed more peroxised than those in control subjects (68) and speculate that changes in membrane structure and function may result, accounting for some of the haemorheological changes observed in diabetes which have been attributed a role in atherosclerosis. A similar suggestion was made by Urano *et al* who showed that $O_2^{\cdot-}$ could induce permeability changes in liposomes reconstituted fron diabetic and non-diabetic erytrocytes and that these changes coincided with increased peroxidation of the lipids (280). They also showed that vitamin E protected the liposomes from peroxidation (280). The function of other cell types involved in atherosclerosis could be altered by similar mechanisms. This could contribute to the abnormal aggregability of platelets in diabetes (54). Perhaps more importantly, lipid peroxides can modify eicosanoid metabolism thus suggesting another mechanism whereby platelet aggregation might be altered by membrane oxidation (111, 281). Related to these suggestions, vitamin E has been shown to reduce diabetic platelet aggregation and production of thromboxane, a pro-aggregatory eicosaniod (282, 125).

Similarly, eicosanoid metabolism in vascular endothelium and smooth muscle may also be involved in the genesis of atherosclerosis (110, 115) and be modulated by vitamin E (281). In addition, glycosylation of proteins in diabetes may have significance in the development of diabetic complications and glycation has been seen to be reduced by vitamin E (103).

7.1.3. Vitamin E Status versus Vitamin E Levels

Diabetes is a condition known to be associated with increased risk of atherosclerosis but the level of vitamin E in diabetes is not clearly known. Platelet levels of vitamin E have been noted to be lower in a group of Type I diabetic subjects than in normal controls (125), but plasma levels were not reported as significantly different (109, 125, 282). This confusion required clarification and might partially have arisen because vitamin E concentrations in plasma were not measured in relation to plasma lipid concentrations. This is important because the concentrations of plasma lipids and vitamin E are highly correlated (145, 259) and a normal vitamin E concentration in the face of raised lipids may in fact indicate deficiency (285) especially in view of the fact that vitamin E protects lipids from oxidation. This notion is confirmed by the finding that resistance of red cells to haemolysis induced by H_2O_2, which can be used as an assay of vitamin E functional activity, correlates better with the ratio of vitamin E to "total lipid" concentrations than with absolute vitamin E concentrations. Since total lipids (triglycerides + cholesterol + phospholipids) are rarely measured, Thurnham investigated the use of other vitamin E:lipid ratios in determining appropriateness of vitamin E level in man (145) and concluded that the tocopherol:cholesterol+triglyceride ratio was almost as powerful as tocopherol:total lipid ratio in detecting vitamin E deficiency (145) therefore in this study I use vitamin E:cholesterol+triglyceride ratios as a measure of vitamin E status.

7.1.4 Summary

Several effects of vitamin E may explain its inverse relationship with the risk of atherosclerosis. One such effect may be its ability to protect PUFA within lipoproteins from peroxidation. It has been shown that the effectiveness vitamin E is better represented by the ratio of the concentrations of vitamin E to those of cholesterol+triglycerides than by absolute vitamin E concentration alone. Diabetic subjects are generally hypertriglyceridaemic but this has not been considered in previous reports of their vitamin E concentration. In this study the ratio of vitamin E concentration to the concentration of cholesterol+triglyceride is used to assess, and is refered to as, vitamin E status and the relationship between vitamin E status and a measure of lipid peroxidation *in vivo*, TBARS, is assessed.

7.2. HYPOTHESIS.

Individuals suffering from diabetes have lower vitamin E levels relative to levels of oxidisable lipid (vitamin E status) than do non-diabetics and lower vitamin E status will be associated with evidence of greater lipid peroxidation in diabetes.

7.3. SUBJECTS AND STUDY DESIGN

36 diabetic subjects were recruited from the routine diabetic clinic in a similar fashion to that described in Chapter 3. Clinical characteristics were obtained, as before, by patient history, supplemented by reference to hospital case notes. Blood samples were lost on one subject, hence results are presented for 35 diabetic subjects. 29 healthy hospital workers volunteered as controls.

7.4. METHODS

Analytical and statistical methods are described in chapter two.

Vitamin E status was determined as the ratio of vitamin E concentration ($\mu mol.l^{-1}$) to the sum of the cholesterol and triglyceride concentrations ($mmol.l^{-1}$)

The concentration of TBARS was used as an estimate of *in vivo* lipid peroxidation.

7.4.1. Assays Performed

Vitamin E

Total Cholesterol (total-Chol)

HDL-Cholesterol (HDL-Chol)

Total Triglycerides (TG)

LDL-Cholesterol (LDL-Chol) by calculation.

TBARS

Fasting plasma glucose (FPG)*

HbA_{1c}*

* in diabetic subjects only

7.5. RESULTS

Diabetic and non-diabetic subjects differed significantly in terms of age, gender but not smoking status (Tab 7.1). The groups also differed in terms of serum lipids. When compared to controls, diabetic subjects were hypertriglyceridaemic (1.60(1.30,2.30) vs 1.10(0.80,1.40) mmol.l^{-1}, p=0.001) and had lower HDL-Chol (1.20(0.35) vs 1.59(0.39) mmol.l^{-1}, p=0.001), whereas their total-Chol and LDL-Chol concentrations were not significantly different (Tab 7.2). Also, in the group as a whole, the men studied were significantly older than the females and had significantly different serum lipids (Table 7.3). In addition, age significantly correlated with total-Chol (r=0.306, p=0.014) and LDL-Chol (r=0.280, p=0.025) and TG (r_s=0.325, p=0.009). Since these differences in age, gender and lipids existed between diabetic and non-diabetic subjects, and interactions between them could occur, they were allowed for statistically using multiple linear regression analysis. Using such analysis to control for age and gender it was confirmed that presence or absence of diabetes was a significant determinant of TG and HDL-Chol concentrations (Table 7.4), that gender significantly influenced HDL-Chol and that age was significantly related to LDL-Chol.

In the group as a whole, vitamin E concentration correlated significantly with total-Chol (r_s=0.545, p=0.001) and TG (r_s=0.353, p=0.004) and total-Chol+TG (r_s=0.601, p=0.001) (Fig 7.1), emphasising the need to consider lipid concentrations, and factors affecting lipids when comparing groups in terms of measures of vitamin E.

Vitamin E concentration was not significantly different in diabetic and non-diabetic subjects (42.6(36.6, 48.8) vs 38.9(32.0, 42.9) μmoll^{-1}, p=0.127). In multiple regression analysis, with logarithmic transformation of vitamin E concentrations, including age, gender, total-Chol, TG, smoking status and presence or absence of diabetes, cholesterol (positively) and age (negatively) were the only statistically significant determinants of vitamin E concentration (Table 7.5a). The relationship with TG concentration just failed to reach statistical significance. This model explained 54.4% of the variability in vitamin E concentration. Removing diabetes, gender and smoking from the model resulted in little change in the percentage variability in vitamin E concentration that was explained (47.1%) (Table 7.5b).

Vitamin E status correlated inversely with, TG (Fig 7.2) and total-Chol+TG (Fig 7.3) in the group as a whole whereas the relationship with total-Chol was not

	Diabetic Subjects	Non-diabetic Subjects	p Value.
Number (n)	35	29	
Age (yrs)	55.9(13.9)	42.7(10.8)	0.002
Sex Ratio (M:F)	26:9	9:20	<0.005 ($\chi^2=18.8$)
Smoking (yes:no)	13:22	8:21	>0.1 ($\chi^2=0.06$)
Duration (yrs)	7.9(2.3,11.5)	-	
Treatment			
Diet	1	-	
Sulphonylurea	23	-	
Sulphonulurea + metformin	0	-	
Insulin	11	-	
Complications			
None	14	-	
Microvascular	5	-	
Macrovascular	4	-	
Micro & Macro	11	-	
Hypertension	1	-	
Fasting plasma glucose (mmol l^{-1})	10.4(4.1)		
HbA_{1c} (%) (NR = 6.5-8.5)	10.1(2.2)		

Table 7.1: Clinical details of subjects.

Values are mean (SD) or median (25th, 75th percentile). Comparisons between groups are tested using unpaired t-tests or chi-squared test.

Lipids (mmol.l^{-1})	Diabetic subjects n = 35	Non-diabetic subjects n = 29	p
Total cholesterol	6.15(1.48)	5.82(0.78)	0.267
LDL-cholesterol	3.88(1.08)	3.72(0.78)	0.484
HDL-cholesterol	1.21(0.35)	1.59(0.39)	< 0.001
Triglyceride	1.60(1.30-2.30)	1.10(0.80,1.40)	< 0.001

Table 7.2: Lipids in diabetic and non-diabetic subjects

Data are expressed as mean(SD) or median(25th,75th percentile) for normally distributed and skewed data resectively. Variables are compaired using Student's t-test for normally distributed data and Mann-Whitney U-test for skewed data.

	Male n=35	Female n=29	p
Age (yrs)	54.9 (13.3)	45.4 (11.9)	0.004
Total cholesterol ($mmol.l^{-1}$)	6.06 (1.39)	5.93 (0.99)	0.651
LDL-cholesterol ($mmol.l^{-1}$)	3.92 (0.98)	3.67 (0.92)	0.312
HDL-cholesterol ($mmol.l^{-1}$)	1.22 (0.33)	1.58 (0.42)	< 0.001
Triglyceride ($mmol.l^{-1}$)	1.60 (1.20,2.15)	1.10 (0.60,1.52)	0.004

Table 7.3: Age and lipids levels in all male and female subjects.

Values are expressed as mean (SD) or median (25^{th},75^{th} percentile) for normally distributed and skewed data resectively. Groups are compared using Student's t-test for normally distributed data and Mann-Whitney U-test for skewed data.

Dependant Variable	Independant Variable	B (95% CI)	p	multiple r^2 n=64
Triglyceride ($mmol.l^{-1}$)	Diabetes	1.09 (0.07,2.11)	0.041	0.105
	Age	0.006 (-0.039,0.044)	0.787	
	Gender	0.03 (-0.07,1.03)	0.948	
Total cholesterol ($mmol.l^{-1}$)	Diabetes	0.05 (-0.64,0.74)	0.879	0.097
	Age	0.01 (0.01,0.05)	0.025	
	Gender	-0.16 (-0.83,0.51)	0.635	
LDL-cholesterol ($mmol.l^{-1}$)	Diabetes	-0.11 (-0.66,0.44)	0.690	0.081
	Age	0.02 (0.001,0.04)	0.046	
	Gender	0.09 (-0.44,0.63)	0.726	
HDL-cholesterol ($mmol.l^{-1}$)	Diabetes	-0.32 (-0.53,-0.11)	0.003	0.308
	Age	0.006 (-0.01,0.04)	0.106	
	Gender	-0.28 (-0.48,-0.08)	0.007	

Table 7.4: The interaction of age, gender and diabetes in determining lipid levels.

Partial regression coefficients (B) plus 95% confidence intervals (95% CI) from a multiple linear regression model including diabetes, age and gender. Male gender coded 1 (Female = 0); presence of diabetes coded 1 (non-diabetic = 0)

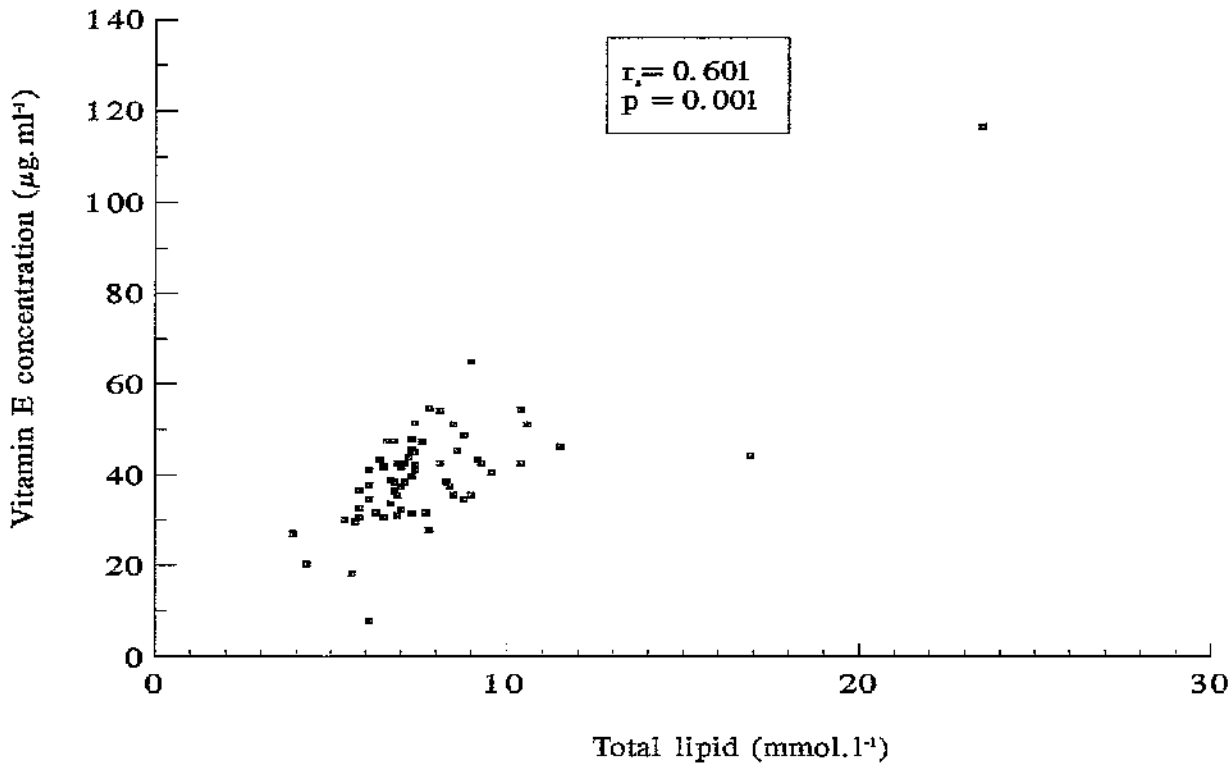


Figure 7.1: The correlation between vitamin E concentration and total lipids (cholesterol concentration plus triglyceride concentration).

Dependant variable	Independant variable	B (95% CI)	p	r^2
a) $\log_e$ vit E	Age	-0.003(-0.001,-0.005)	0.015	0.544
	Gender	-0.002(0.058,-0.061)	0.957	
	t-Chol	0.094(0.122,0.065)	<0.0001	
	TG	-0.006(0.013,-0.013)	0.519	
	Diabetes	0.051(0.115,-0.013)	0.118	
	Smoking	-0.033(0.022,-0.088)	0.229	
b) $\log_e$ vit E	Age	-0.002(-0.0003,-0.004)	0.027	0.471
	t-Ch	0.086(0.114,0.059)	<0.0001	
	TG	0.001(0.019,-0.017)	0.938	
c) Vit E status	Age	-0.06(-0.11,-0.003)	0.037	0.128
	Gender	-0.38(-1.83,0.12)	0.606	
	Diabetes	0.31(-0.22,1.06)	0.686	
	Smoking	-0.84(-2.20,0.52)	0.223	

Table 7.5: The determinants of vitamin E concentration and vitamin E status

Partial regression coefficients (B) plus 95% confidence intervals (95% CI) and multiple r^2 values are given for multiple linear regression models including and excluding those independent variables listed.

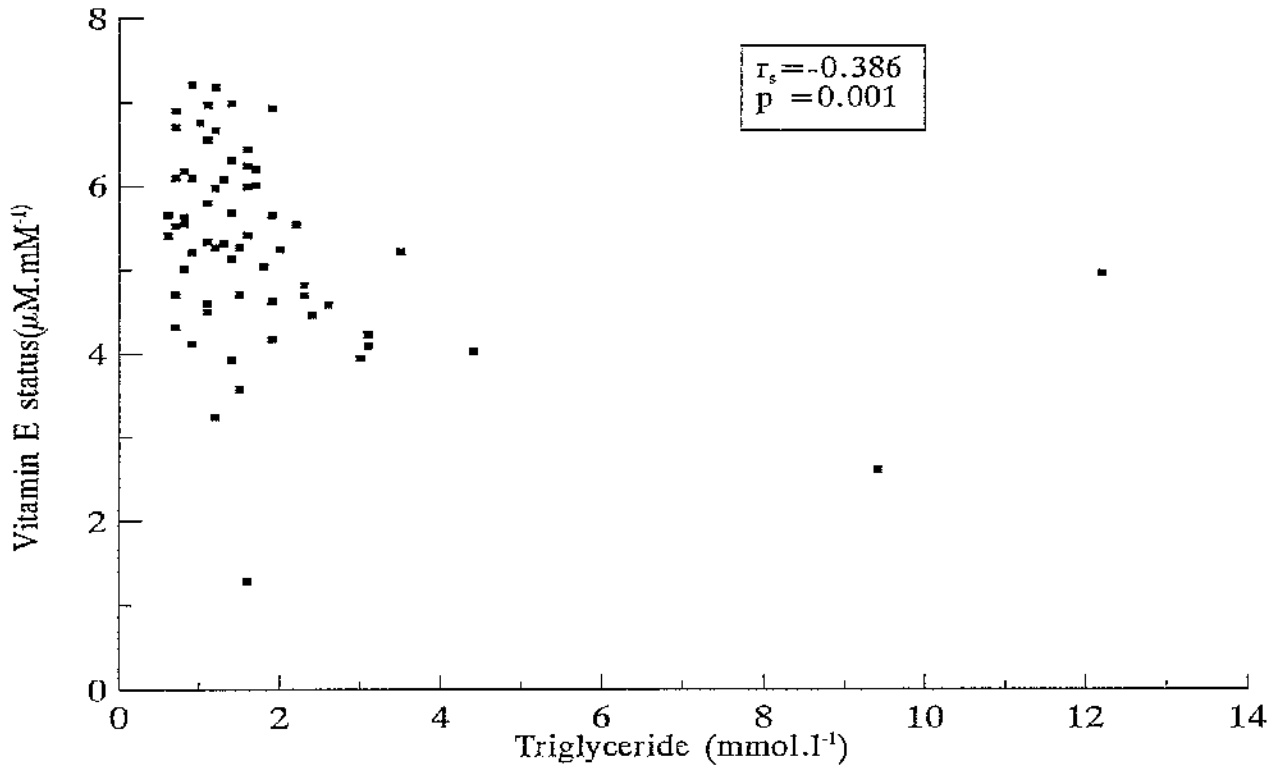


Figure 7.2: The correlation between vitamin E status and triglyceride concentration.

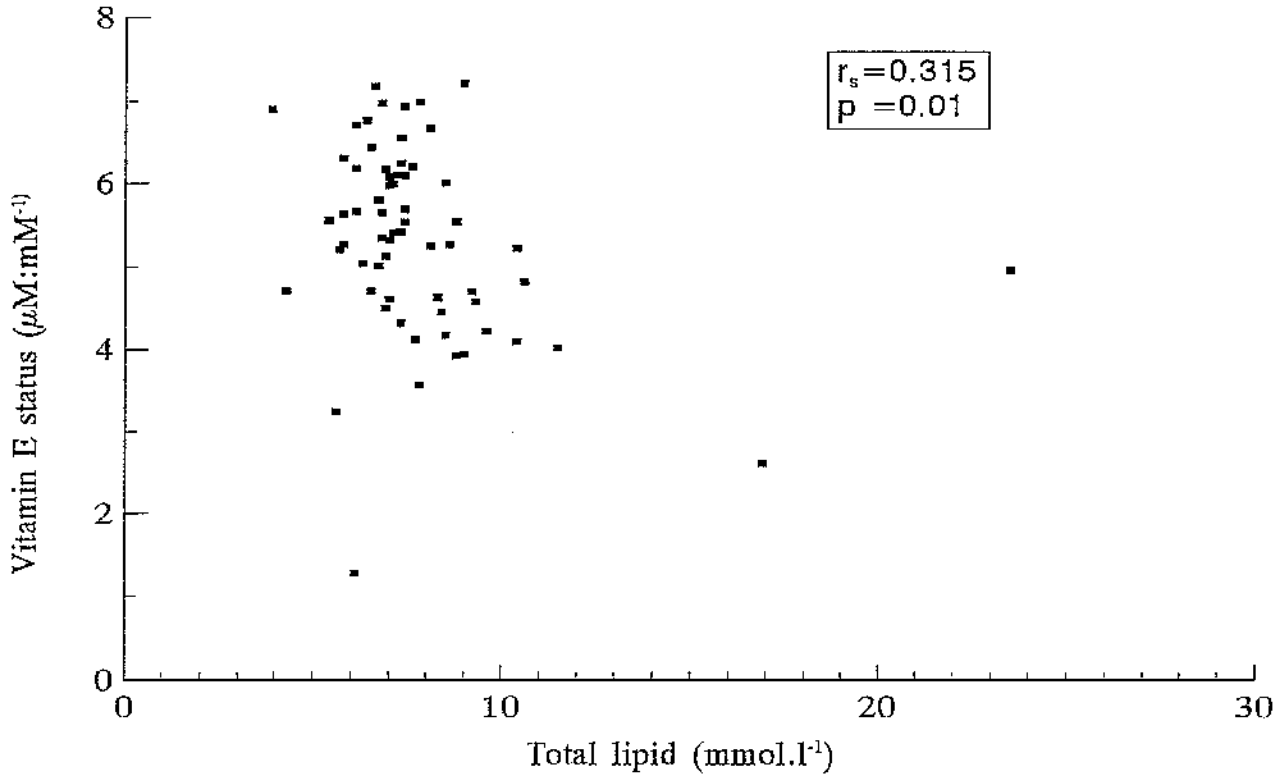


Figure 7.3: The correlation between vitaminE status and
total lipids (cholesterol concentration plus triglyceride concentration)

statistically significant (Fig 7.4). Consequently in those individuals with high total-Chol (total-Chol $\geq$5.8mmol.l^{-1}) vitamin E status was significantly lower than in those with total-Chol $\leq$5.8mmol.l^{-1} (5.67(1.22) vs 5.08(1.08) μM.mM^{-1}, p=0.047) also in those with TG $\geq$ 1.8 mmol.l^{-1} compared to those with TG $\leq$ 1.8mmol.l^{-1} vitamin E status is significantly lower (5.56(1.14) vs 4.71(0.09) μM.mM^{-1}, p=0.003). Vitamin E status was not significantly different in diabetic and non-diabetic subjects (5.19(1.22) vs 5.48(1.03) μM.mM^{-1}, p=0.309) (Fig 7.5). In a multiple linear regression model including age, gender, diabetes and smoking, age was significantly, inversely correlated with vitamin E status (Table 7.5c). Vitamin E status did not correlate with plasma glucose or HbA_{1c} in diabetic subjects (Tab 7.6).

TBARS concentration was significantly greater in diabetics than in non-diabetics (2.40(0.69) vs 1.67(0.63) μmolMDA.l^{-1}, p<0.001) (Fig 7.6). Also, TBARS concentration did not correlate significantly with vitamin E status (r= -0.117, p=0.358).

In a multiple linear regression analysis including age, gender, smoking, diabetes and vitamin E status, diabetes was confirmed as a highly significant determinant of TBARS concentration (Table 7.7). However in diabetic subjects, TBARS did not significantly correlate with HbA_{1c} but did statistically significantly correlate with plasma glucose and duration of diabetes (Table 7.6).

TBARS concentration significantly correlated with TG (r_s=0.338, p=0.006) (Fig 7.7) but not total-Chol (r=0.171,p=0.177). However, in multivariate analysis in which diabetes, smoking and age, were allowed for the relationship between TBARS and TG concentration failed to reach significance (Table 7.7b).

In diabetic subjects neither vitamin E status nor TBARS concentration were significantly different in those with and without complications (Table 7.8) and correction for age, gender and smoking did not alter this result.

7.6. DISCUSSION

In agreement with other studies, vitamin E levels were found to correlate with total-Chol, TG, LDL-Chol and the sum of total-Chol + TG (145). When assessing factors which may influence vitamin E concentration it is necessary to consider whether or not those factors do so independently or simply through their confounding effects on lipids. Age, gender and diabetes are known to influence serum lipid profiles (38, 284, 285). The effect of diabetes on lipid profiles,

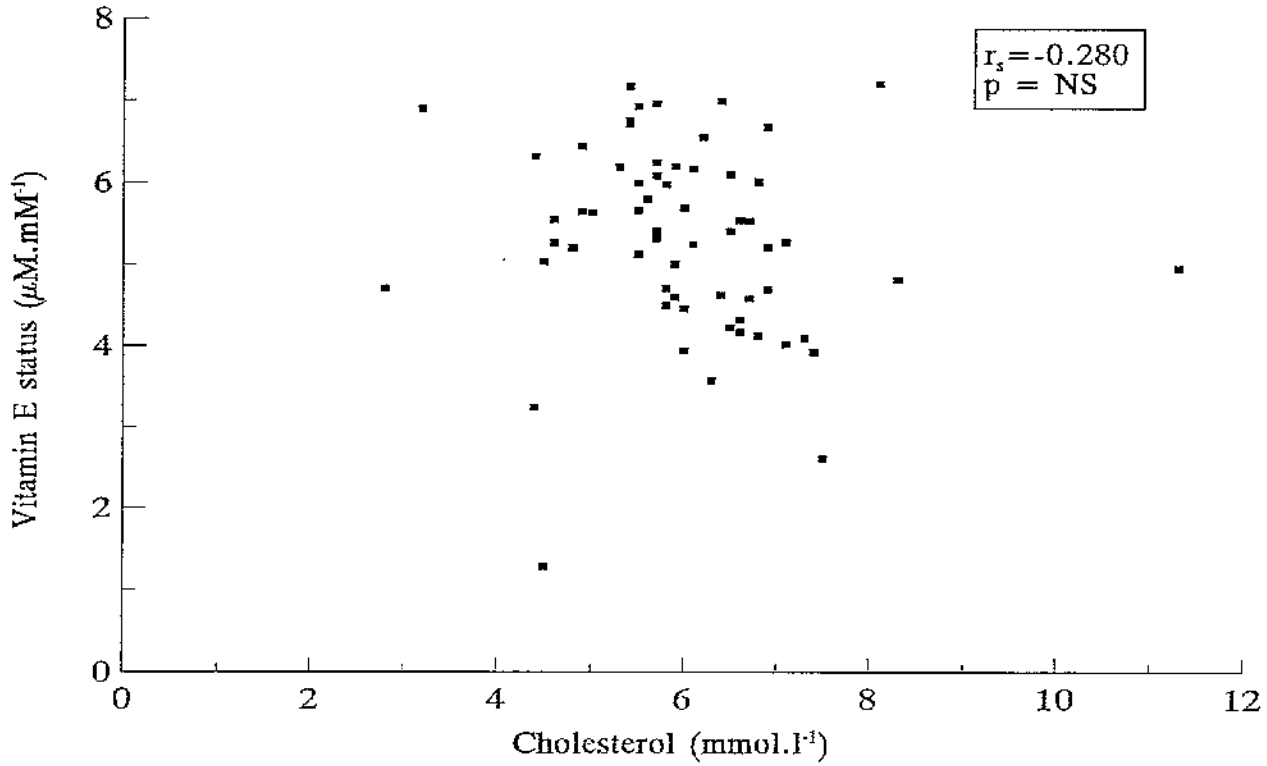


Figure 7.4: The correlation between vitamin E status and cholesterol concentration.

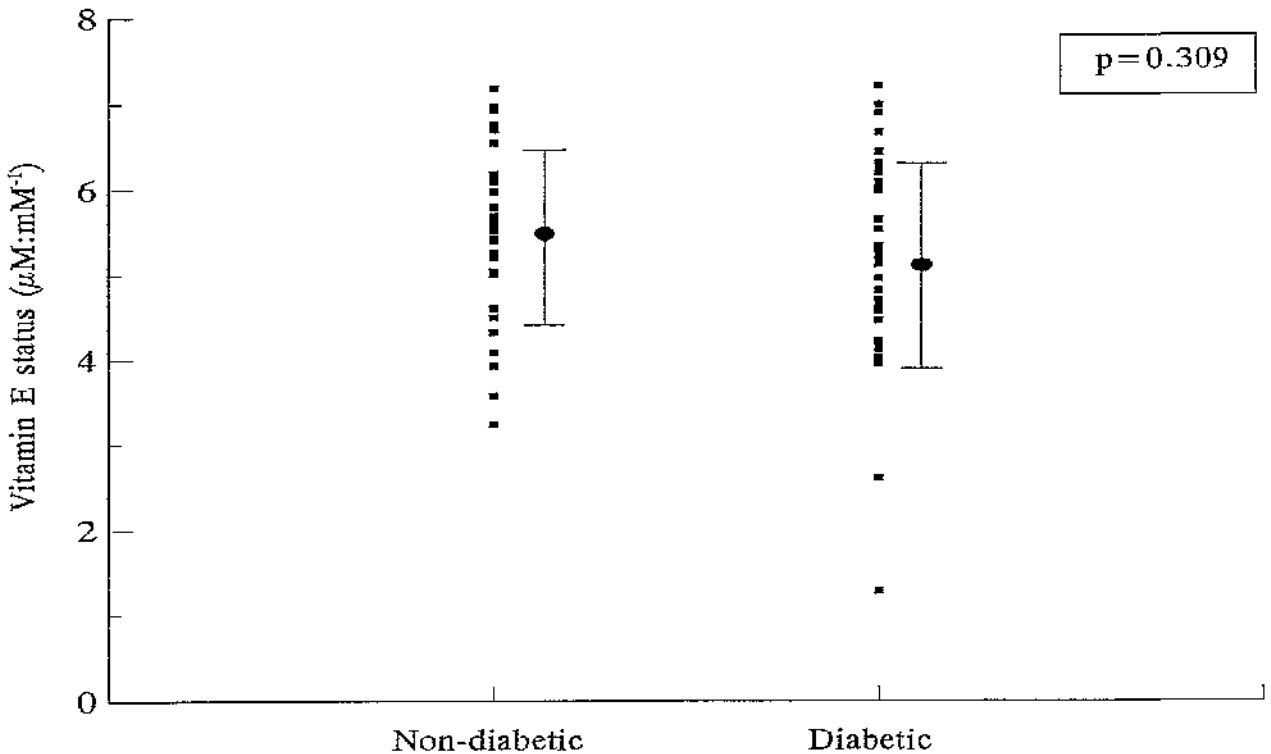


Figure 7.5: Vitamin E status in non-diabetic and diabetic subjects.

	Glucose		HbA_{1c}		Duration	
	r	p	r	p	r_s	p
TBARS (μmol.l^{-1}) (n=34)	0.394	0.021	0.155	0.383	-0.440	0.008
Vit E status (μmol:mmol) (n=34)	-0.290	0.097	-0.165	0.350	0.015	0.934

Table 7.6: Correlation of vitamin E status and lipid peroxidation with glucose, HbA_{1c} and duration of diabetes in diabetic subjects.

Pearson correlation coefficients (r) or Spearman rank correlation coefficients (r_s) are given.

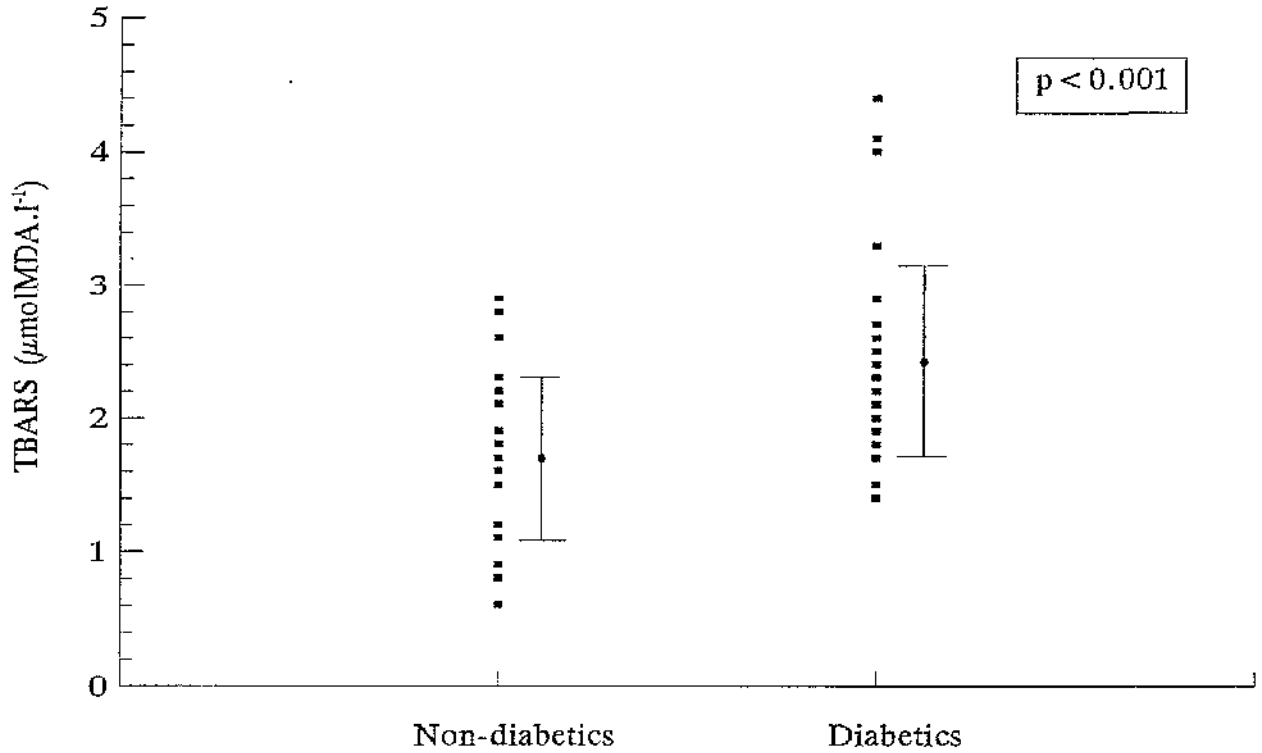


Figure 7.6: Thiobarbituric acid reactive substances in non-diabetic and diabetic subjects.

Dependant variable	Independant variable	B (95% CI)	p	r
a) TBARS (μmol.l^{-1})	Age	-0.01(-0.03,0.003)	0.066	0.319
	Gender	-0.15(-0.52,0.23)	0.440	
	Smoking	0.26(-0.08,0.99)	0.142	
	Diabetes	0.91(0.53,1.30)	0.000	
	Vit E status	-0.03(-0.10,0.04)	0.383	
b) TBARS (μmol.l^{-1})	Age	-0.01(-0.03,0.00)	0.048	0.343
	Diabetes	0.76(0.39,1.13)	0.000	
	Smoking	0.30(-0.03,0.64)	0.081	
	TG	0.09(-0.006,0.18)	0.066	

Table 7.7: Determinants of lipid peroxidation as assessed by multiple linear regression analysis.

Lipid peroxidation was determined as the concentration of thiobarbituric acid reactive substances (TBARS). The partial correlation coefficient (B) and 95% confidence intervals (95% CI) from a multiple linear regression model. The multiple r (r) value shown.

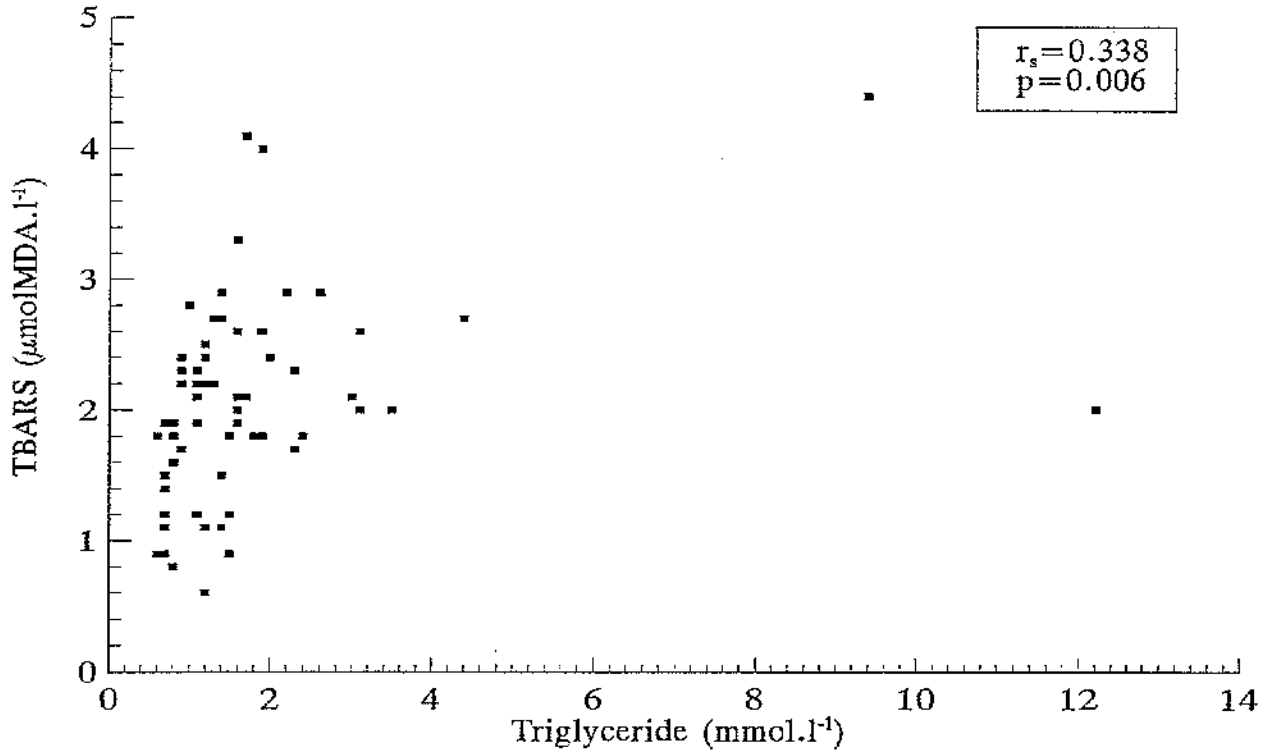


Figure 7.7: The correlation between thiobarbituric acid reactive substances and triglyceride concentration.

	With Complications n=21	Without Complications n=14	p
TBARS (μmol.l^{-1})	2.28(0.66)	2.57(0.72)	0.238
Vit E status (μmol:mmol)	11.28(3.3)	12.94(1.62)	0.054

Table 7.8: Lipid peroxidation and vitamin E status in those diabetic subjects with and without complications.

Data are expressed as mean (SD) and comparisons made by Student's t-test.

independent of age and gender, was confirmed in this study; diabetic subjects were hypertriglyceridaemic and had lower HDL-Chol than normal controls (82). Smoking has also been associated with alterations in lipids and lipoproteins (191) but the proportion of smokers in the diabetic group was not significantly different from that in the non-diabetic group. The age and gender of the diabetic and non-diabetic subjects differed significantly in this study but this was controlled for by multiple linear regression analysis.

In addition to an effect mediated by lipids, age and gender may have direct effects on vitamin E levels, but this is debated (257). The confusion may arise because the confounding effects of lipid alterations have not always been considered. Similarly, the possible confounding effect of lipid levels was not considered in reports of normal plasma vitamin E levels in diabetes (125). Smoking has been shown to be associated with the reduced ability of erythrocytes to resist peroxidation, a defect which can be corrected by vitamin E supplementation, however although differences in other antioxidants were reported (195, 197) no difference in plasma vitamin E levels has been noted between smokers and non-smokers (194, 197).

Taking into account the possibility of confounding influences of age, gender, TG, total-Chol and smoking I have shown in this study that vitamin E levels are not significantly influenced by diabetes. In fact, in a multivariate regression analysis, serum total-Chol and age were the significant determinants of vitamin E, age being inversely related to vitamin E levels. The reasons for this effect of age are not known but reduced intake, absorption, altered metabolism or increased consumption are all possibilities. Interestingly, although vitamin E concentrations and TG do correlate significantly in univariate analysis, they do not in this multivariate model. Another way to allow for lipid concentrations whilst assessing vitamin E concentrations is to determine the ratio of vitamin E concentration to lipid concentrations. Justification for correcting for lipids in this way is the finding that such a ratio has functional relevance in that the ratio correlates better with the inhibition of H_2O_2 induced erythrocyte haemolysis than does vitamin E concentration alone (145). Using the vitamin E to total-Chol+TG ratio to assess vitamin E status in this study I found that this ratio was not significantly lower in diabetic subjects than non-diabetics and in multivariate regression analysis, diabetes was not found to be a significant determinant of vitamin E status. Again, age was significantly inversely related to the ratio but the correlation was weak. In keeping with the observation that diabetes mellitus has no effect on vitamin E status is the lack of

correlation between plasma glucose, HbA_{1c} and duration of diabetes with Vitamin E status in diabetic subjects.

I feel it is worth commenting on the behaviour of the vitamin E status with regards to lipids. Although mathematically obvious it may have physiological significance. Vitamin E concentration increases with total-Chol and TG levels, however, they do not rise in parallel if they did then, as total-Chol and TG rose, the ratio of vitamin E to total-Chol and TG , in other words the vitamin E status, would remain the same, it does not. The ratio is actually significantly inversely related to TG concentration (Fig 7.3), and not related to total-Chol concentration (Fig 7.4). Approached in a slightly different way, this means that at higher total-Chol and TG levels, the ratio is lower and so in hypertriglycerideaemic and hypercholesterolaemic subjects the vitamin E status is significantly lower than in normolipidaemic individuals. Several models of how vitamin E concentrations vary with lipids could explain this phenomenon but the numbers here are too small to establish which. Vitamin E could rise linearly throughout the entire range of lipid concentrations with a slope to the regression line of less than 1 or a saturatable system could exist where the relationship plateaus beyond a certain level of lipids. No matter what the model however ,if a certain amount of vitamin E is required to protect a certain amount of lipid the outcome remains that at higher lipid levels relatively too little vitamin E may be availible. Perhaps another relevant point is that in multiple regression analysis of the determinants of vitamin E concentrations, total-Chol was much more significantly associated than was TG and the slope of the relationship much steeper with t-Chol (3.45) than with TG (0.54) (Table 7.5). In other words, a rise in TG may be less closely matched by a rise in vitamin E than is a rise in total-Chol.

Overall, the implication is that with hyperlipidaemia there may be a mismatch between Vitamin E and the lipids it protects and the mismatch may be greatest in hypertriglyeridaemic states. This may be particularly relevant in diabetes where hypertriglyceridaemia is a common phenomenon and may be an independent cardiovascular risk factor (82, 286, 287).

In vivo lipid peroxidation as estimated by plasma TBARS was found to be significantly greater in this group of diabetic subjects compared to controls, in agreement with other reports on diabetic animals and humans (65-69, 288) and is consistent with the hypothesis that more FR lipid peroxidation occurs in diabetes. Further supporting an effect of diabetes, TBARS correlated with plasma glucose,

although not with HbA_{1c}. Jain *et al* found the TBARS content of human diabetic erythrocyte (RBC) membranes did correlate with HbA_{1c} but did not measure plasma glucose (68). My study and that of Jain *et al* are in agreement in so far as they both imply that glycaemia may influence peroxidation *in vivo*. The different relationship observed with HbA_{1c} may reflect differences the half-life of the peroxidised amterial studied. The TBARS in RBC membranes may be more long lived than those in plasma and so reflect the average glucose exposure over a period of time. HbA_{1c} also reflects the average glucose exposure during the live of an erythrocyte hence the observed correlation between the two. Short lived plasma TBARS may be more influenced by the immediate ambient plasma glucose.

The mechanism whereby diabetes might cause an elevation in plasma TBARS is not clear but was discussed in Section 6.6.2. As argued there although the presence of glucose has been shown to enhance the peroxidation of human isolated LDL (100) this direct effect of glucose may not be the only explanation for the raised TBARS in diabetes and in that section I offered some possible explanation for why I and others have foung that peroxidisability of plasma was unrelated to levels of glycaemia (Section 6.6.2) despite the enhanced peroxidation of LDL on exposure to glucose and elevated TBARS in diabetics.

The effect of diabetes on TBARS persisted even after controlling for age, gender, smoking and vitamin E status. Vitamin E status had no significant relationship with TBARS in this multivariate, or in univariate, analysis. This is contrary to the hypothesis that lipid peroxidation is inversely related to vitamin E status and other factors must be involved in determining plasma TBARS. The possible inaccuracies in using TBARS as an estimate of *in vivo* lipid peroxidation have already been discussed (Section 6.1). In addition, there is a great inter-individual variability in the PUFA content of cholesterol esters and triglycerides and it may be that vitamin E status assessed relative to total-Chol and TG is too crude and it needs to be assessed relative to oxidisable PUFA concentration or even more specifically to vulnerable double bonds.

In univariate analysis TBARS concentration appeared to correlate significantly with TG which would have been in agreement with the findings in streptozotocin diabetic rats as discussed by Chisolm (288). No such correlation was found with total-Chol. Such a relationship could simply reflect the fact that the TBARS assay cannot detect the lipid hydroperoxide of linoleic acid, the major fatty acid esterified to cholesterol (28) however it is also consistent with the suggestion that triglycerides may be more

vulnerable to oxidation *in vivo* and so suggest a mechanism to explain the risk of vascular disease associated with hypertriglyceridaemia (286). A further connection between TG concentration and lipid oxidation is made by the interesting finding that the resistance of isolated LDL to oxidation *in vitro* is inversely related to the TG content of LDL (140). However in the current study the relationship between TBARS and TG concentrations just failed to maintain statistical significance in a multiple regression model (Table 7.7). If a relationship does exist between TBARS and TG this might appear to contradict the finding in chapter six that peroxidisability (change in tDC not TBARS) correlated with total-Chol and not TG. This apparent contradiction may reflect the fact that TBARS and peroxidisability measure different things. TBARS estimates lipid peroxides present in plasma at a given time and reflects a balance between their production and removal *in vivo* whereas peroxidisability measured the change in tDC over a period of time *in vitro*. However any contradiction does add further to the doubts discussed in Section 6.6.1c regarding the interpretation of *in vitro* assays of peroxidisability.

7.7 CONCLUSION

This study did not support the hypothesis that vitamin E status is lower in diabetes and vitamin E status did not correlate with levels of lipid peroxidation *in vivo*. In addition despite normal vitamin E status, the level of lipid peroxidation was found to be greater in diabetes, confirming previous reports, and further implying that the increased lipid peroxidation which occurs in diabetes, and which may help explain the increased risk of cardiovascular disease in diabetes, is not significantly influenced by vitamin E status. However, age was found to be inversely related to vitamin E status.

Because vitamin E levels do not rise in parallel with lipids, and this is particularly so for triglycerides, then in hyperlipidaemic states, especially hypertriglyceridaemia, significant mismatch between vitamin E and oxidisable lipid may arise. This mismatch may result in an increased tendency to lipid oxidation at high lipid levels.

CHAPTER EIGHT

THE EFFECT OF VITAMIN E AND VITAMIN C SUPPLEMENTATION ON PLASMA TOTAL PEROXYL RADICAL ANTIOXIDANT ACTIVITY, VITAMIN E STATUS AND LIPID OXIDATION

8.1 INTRODUCTION

The study described in this chapter follows on from the background, results and discussion of the previous chapters. To recapitulate briefly, there are theoretical grounds for expecting that antioxidant vitamins might reduce oxidation of LDL and consequently reduce the development of atherosclerosis. In keeping with this view are epidemiological studies correlating risk of morbidity and mortality from vascular disease with reduced plasma levels of antioxidant vitamins (198, 199, 279). I have shown that diabetes interacts with smoking such that total peroxyl radical antioxidant activity of plasma (TRAP) in diabetic smokers is lower than in non-smoking diabetics and in healthy controls. Both diabetes and smoking are important risk factors for atherosclerosis. However, I found no correlation between TRAP and plasma lipid peroxidisability *in vitro*. Also vitamin E status was not statistically significantly lower in diabetics than in control subjects and again I found no correlation between vitamin E status and TBARS, an estimate of lipid peroxidation *in vivo*. Nevertheless there was evidence of increased peroxidation in diabetes provided by the finding of greater TBARS concentration in diabetics than non-diabetics

Others have also been unable to demonstrate actual correlation between measures of antioxidants and of lipid peroxidation assessed in several ways (27, 28, 46, 143, 255). Despite this, it has been found that increasing the antioxidant content of LDL does increase the resistance of LDL to peroxidation when subjected to various oxidative stresses *in vitro* (28, 46). There are many possible explanations for this apparent contradiction and these have been discussed at length elsewhere (6.5.1), but it seemed possible that a correlation between antioxidant levels and peroxidation might better be demonstrated by assessing the effects of changes in antioxidant levels upon changes in measures of peroxidation within an individual. Hence, I aimed to assess the effect of both vitamin C and vitamin E supplementation on antioxidant activity and lipid peroxidation. Because studies in diabetic rats and humans, which suggested that

ascorbate metabolism may be abnormal in diabetes (119, 122), implied that supplementation in diabetics and non-diabetics might not have the same effect both diabetic and non-diabetic subjects were studied.

Plasma TBARS were measured as an estimate of *in vivo* lipid oxidation. The susceptibility of isolated LDL to oxidation was measured using a method described by Esterbauer (213), the principle of which is described below. The measures of antioxidants employed in this study were TRAP, vitamin E status and uric acid levels. TRAP measures total activity as opposed to concentration and thus will take into account effect of interactions between antioxidants in plasma. I will discuss the role of uric acid as an antioxidant in section 8.1.2 but it was measured here because its levels have been reported to be abnormal in diabetes and the antioxidant activity of vitamin C may be closely associated with that of uric acid. In addition uric acid is a major contributor to TRAP and therefore I expected that their levels should correlate. The method available for measuring uric acid is more robust than the TRAP assay and confirmation of a correlation between TRAP and uric acid would help validate TRAP measures.

The ultimate aim was to study four groups;-

a) healthy controls
b) non-diabetic individuals with macrovascular disease
c) diabetic individuals without complications
d) diabetic individuals with macrovascular disease.

The study reported here was the first phase and was to act as a pilot for the full study. I began by looking at groups a) and d), as I anticipated that if differences were going to exist they would be maximum in these groups therefore it is for these groups that results are reported here.

8.1.1 The Oxidation Of Isolated Low Density Lipoprotein

The method employed was a modification of that described by Esterbauer (213). The precise experimental detail is given in chapter two. LDL is isolated from plasma by ultracentrifugation and its *in vitro*, copper stimulated oxidation studied. Oxidation of LDL during the isolation procedure is inhibited by use of betahydroxytoluene (BHT) and EDTA throughout, by performing manipulations on ice and by storage at 4°C, in the dark. BHT and EDTA are removed, only immediately before use, by dialysis

against degassed buffers.

Oxidation of isolated, dialysed LDL is then stimulated by addition of copper ions. The reactions outlined in Fig 3.1 occur and the process can be followed continuously by observing the rise in absorbance at 234nm due to the appearance of diene conjugated species (DC). A typical trace is shown diagrammatically in Fig 8.1. and reveals three phases of oxidation (213). During the first phase, known as the lag phase, peroxidation is essentially inhibited and minimal rise in absorbance occurs. It has been established that during the lag phase some of the antioxidants in LDL,including vitamin E, gradually disappear and it is believed that the lag phase terminates when insufficient antioxidant remains to prevent peroxidation. However the duration of the lag phase (LAG) has not been shown to correlate with the antioxidant content of LDL and so LAG cannot be solely dependant upon antioxidants (28, 46). The second phase of LDL oxidation is called the propagation phase and during it peroxide and non-peroxide diene conjugated species form and so absorbance increases. Factors influencing the rate of peroxidation (DC-RATE) and the extent of peroxidation ($DC.mg^{-1}$) are unknown as is the reason why PUFA oxidation stops when only a fraction of the potentially oxidisable moieties have reacted (143). When peroxidation ends phase three is entered and a plateau in absorbance is reached. Decomposition of labile oxidation products is the major event identified in this phase. An oxidation experiment allows the following measurements to be made from the absorbance curve:-

1. duration of the lag phase (LAG) (minutes)
2. the change in 234nm absorbance reflecting change in total diene conjugated species concentration (ΔAb)
   (arbitrary units)
3. duration of propagation phase (PROP) (minutes)

Since the change in absorbance (ΔAb) directly relates to the rise in concentration of diene conjugated hydroperoxides and the extinction coefficient (ϵ_{234}) for such species is 29,500 $M^{-1}.cm^{-1}$. (213) the change in concentration of diene conjugated hydroperoxide (DC-CONC) can be calculated as follows

$$DC\text{-}CONC = \frac{\Delta Ab}{\epsilon_{234}} \ mol.l^{-1}$$

Since the LDL concentration used was 0.25×10^3 $mg.l^{-1}$ (0.25 $mg.ml^{-1}$) the amount of

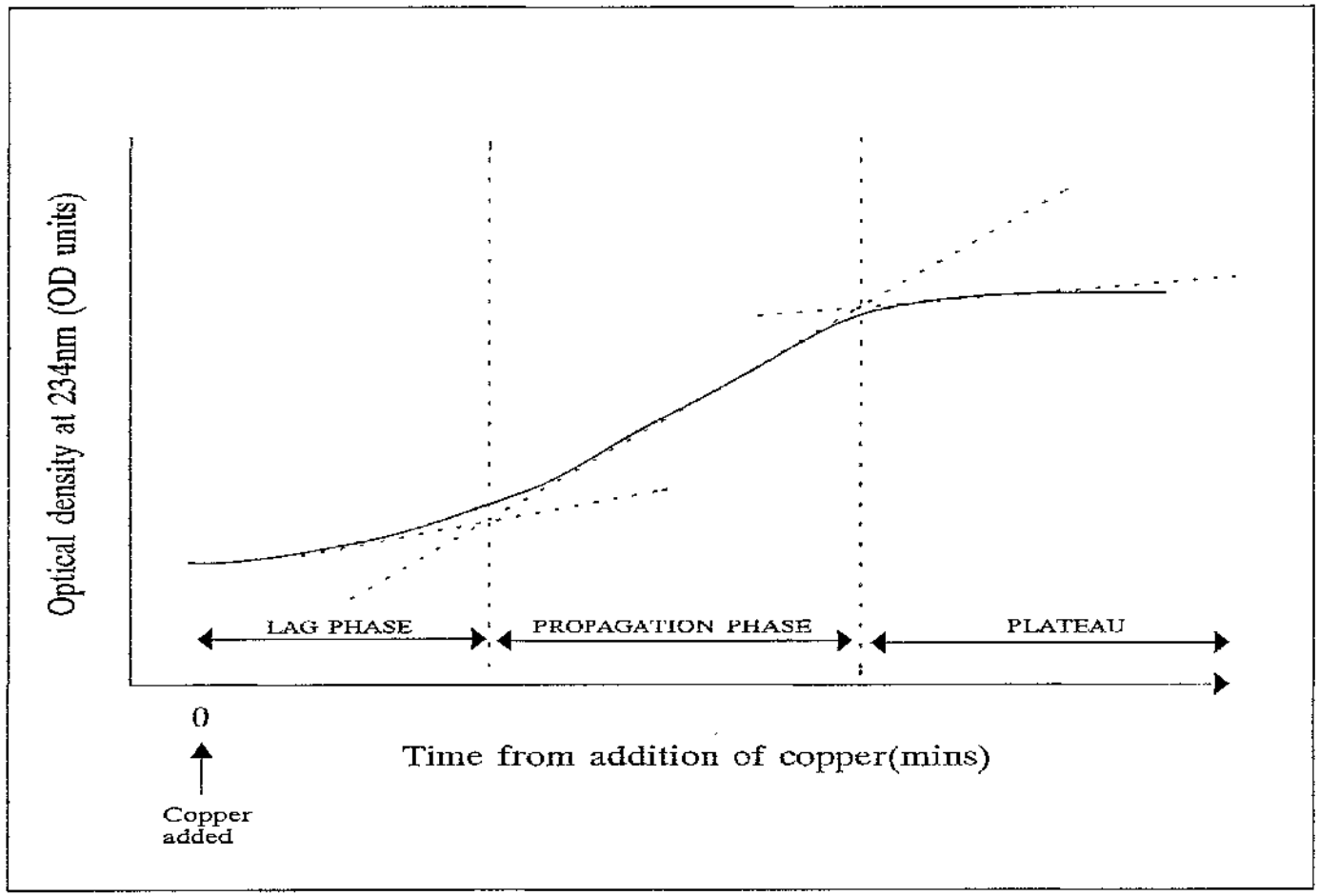


Figure 8.1: A representation of a typical trace of optical density versus time obtained during *in vitro* copper stimulated oxidation of low density lipoprotein.

diene conjugated material formed per mg of LDL ($DC.mg^{-1}$) is

$$DC.mg^{-1} = \frac{\Delta Ab}{\epsilon_{234} \times 0.25 \times 10^{3}} \text{ mol.DC per mg LDL}$$

$$= \frac{\Delta Ab \times 4 \times 10^{6}}{\epsilon_{234}} \text{ nmol.DC per mg}$$

Knowing the duration of the propagation phase (PROP) then the rate of formation of diene conjugates (DC-RATE) can also be calculated

$$DC\text{-}RATE = \frac{\Delta Ab \times 4 \times 10^{6}}{\epsilon_{234} \times PROP} \text{ nmol.DC.mg}^{-1}.\text{min}^{-1}$$

Thus several aspects of LDL oxidation *in vitro* can be measured however the only aspect shown to have any pathological correlate so far is the LAG. As discussed in chapter six the lag phase of the *in vitro* oxidation of LDL isolated from survivors of a myocardial infarction was shown to correlate negatively with the extent of atherosclerosis seen at angiography (140). Why the LAG might be important is not known but theoretically may be because the longer the lag phase the less likely it is that LDL within plasma or subendothelial sites will be oxidised to more atherogenic forms before it can be removed. Thinking along similar lines once LDL starts to oxidise, the faster it does so the more likely it will be that it is sufficiently oxidised to be atherogenic before removal. This suggests one mechanism whereby rate of peroxidation might also be relevant in terms of atherogenesis. Also the rate at which LDL oxidises and the extent to which it can do so will help determine how much oxidised material exists at any one time. If direct toxicity of lipid hydroperoxides is important in atherogenesis then rate of peroxidation (DC-RATE) and extent of peroxidation ($DC.mg^{-1}$) may also be important considerations regarding atherogenesis. Since all of these features of the peroxidation process could theoretically influence atherogenesis I have investigated the effect of antioxidant supplementation on the rate and extent of peroxidation in addition to the duration of the lag phase. Although the LAG is the feature of peroxidation most frequently studied interest is developing in some of the other features. For example, the rate of propagation was measured in addition to LAG by Cominacini *et al* who used the development of fluorescence to assess the predisposition to copper stimulated oxidation of isolated human LDL (289) and its

relation to α-tocopherol content. Interestingly they found that the rate of propagation and LAG were significantly correlated suggesting that factors which influence one may influence the other.

8.1.2 Uric Acid As An Antioxidant.

The final product of purine metabolism is uric acid which is ultimately excreted predominately via the kidney (290). However uric acid can also function as an antioxidant (13) and there is evidence that uric acid is produced in certain circumstances over and above any simple need to remove purines. For example, studies of arteriovenous differences in uric acid levels employing cathers in the carotid sinus and coronary arteries of human hearts, showed that more uric acid is drained from the heart than can be explained by the uptake of purines (291). Assuming that purine balance is maintained this implies that the heart either produces more purine than it requires, or that the production of uric acid is intended. Also the major antioxidant present in the secretions which are released from nasal submucosal glands in response to acetyl choline has been characterised as uric acid (292).

Uric acid, when added to plasma, was found to prolong the lag phase before lipid peroxidation began (26) when the FR generator was the water soluble ABAP. Added to isolated LDL it can also prolong the lag phase before transition metal catalysed oxidation begins (213). In parallel with these observations urate present in plasma is consumed on exposure to FR sources (27). These observations serve to support the impression that uric acid is a radical scavenger and antioxidant. In fact Wayner *et al* calculated that at the concentrations found in plasma urate would contribute between 35% and 60% of the plasma TRAP (26). Uric acid may function as antioxidant in a variety of ways. It may act as a chain breaking antioxidant by reducing radicals, it may chelate transition metals and so act as a preventative antioxidant and it may interact with ascorbate to prevent its auto-oxidation hence both preserving ascorbate as an antioxidant and interfering with vitamin C's pro-oxidant activity (Section 8.6.2) (29).

The effect of diabetes upon uric acid levels is debated. Some groups have reported no difference in plasma uric acid concentrations in IDDM or NIDDM patients compared to controls (293) whilst others found hypouricaemia in NIDDM (294) and in IDDM which may be explained by a renal tubular defect in urate reabsorption (295, 296) secondary to increased reabsorption of glucose. Conversely hyperuricaemia has been reported in prediabetes (294) and in association with hypertension,

hypertriglycerideaemia, insulin resistance and hyperinsulinaemia (296) features all common in non-insulin dependent diabetes. In fact uric acid concentration has been reported to be a predictor of hypertension (298) and a risk factor for coronary artery disease (202). The lack of consensus regarding the influence of diabetes on uric acid concentrations may arise because diabetes has heterogenous effects on uric acid levels depending upon such features as the degree of hyperglycaemia and renal function.

8.2 HYPOTHESIS

Vitamin C and vitamin E dietary supplementation will increase TRAP and vitamin E status of plasma respectively and reduce lipid peroxidation in diabetic and non-diabetic subjects.

8.3 SUBJECTS AND STUDY DESIGN

Ten non-insulin dependent diabetic (NIDDM) subjects with macrovascular disease (as previously defined) were recruited. One subject dropped out for non-medical reasons and so results presented are for nine NIDDM subjects. Ten healthy laboratory workers volunteered as controls. The clinical characteristics of all subjects are given in Table 8.1.

Subjects attended, after an overnight fast, on 4 occasions. Subjects were assessed at baseline (Visit 1) and then asked to take 1g of ascorbic acid (vitamin C) daily for 3 weeks at which time they were reassessed (Visit 2). Following a 3 week washout period in which no vitamin supplements were taken subjects were studied for a third time (Visit 3) and prescribed 300mg tocopherol acetate (vitamin E) daily for 3 weeks when they were seen for final assessment (Visit 4). Subjects were asked to continue their normal diets throughout the study period. Diabetic subjects continued their normal medication.

		Diabetic Subjects	Non-diabetic Subjects	p value
n		9	10	
Age	(yrs)	61.1(12.9)	41.5(10.8)	0.003
Sex Ratio	(M:F)	8:1	7:3	$p > 0.2$ $\chi^2 = 1.03$
Duration	(yrs)	9.4(7.6)	N/A	
Smoking	(yes:no)	3:6	1:9	$p > 0.2$ χ^2
Treatment Diet Sulphonylurea + Metformin		5 4		
Complications Macrovacsular Microvascular		9 2		

Table 8.1: Clinical details of subjects

Values are mean (SD). Groups are compaired by unpaired Student's t-test of Chi-squared test.

8.4 METHODS

Analytical methods were as described in chapter two and vitamin E status was calculated as before (Section 7.3.2). The statistical methods used to compare groups at baseline were as described in chapter two. The difference in age between the groups was allowed for using analysis of variance with age as a covariate.

To analyse the effects of vitamin supplementation it had to be taken into account that the design of this study was such that, within a subject, the same variable was measured repeatedly (4 occasions). Hence a statistical approach capable of allowing for this repeated measures design was employed namely *the repeated measures analysis of variance* as available within the SPSS computer program (299). This technique tests the hypothesis that there is no difference in variables over the four measurements made. In the context of this study the hypothesis becomes that there is no overall effect of the treatments used. In addition to allowing for repeated measures use of this statistical method also reduces the number of paired tests performed and so the probability of false positive results. The effect of diabetes, and the covariate age, on the treatment effect were included in the analysis. Variance ratios (F) were determined for the overall treatment effect and for the influence of diabetes and age on the treatment effect. Since including age as a covariate did not significantly alter any of the outcomes observed only the F ratios for treatment and diabetes effects are quoted. Where a significant overall effect was identified the specific effect of vitamin C or vitamin E was assessed using paired t-tests or Wilcoxon sign rank test. The effect of vitamin C was determined by comparing results from visits 1 and 2 and of vitamin E by comparing visits 3 and 4. Where results were missing for visit 3, visit 1 results were used if there was no significant difference in the variable between visits 1 and 3 in the rest of the group.

8.4.1 Assays Performed

Fasting plasma glucose (FPG).

HbA_{1c}.

total-Chol, TG, HDL-Chol - with calculation of LDL-Chol.

TRAP.

TBARS.

Oxidation of isolated LDL with determination of LAG and PROP and calculation of DC-Rate and DC.mg^{-1}.

Uric Acid.

Vitamin E - with calculation of vitamin E status as the ratio of vitamin E concentration to that of the total-Chol+TG concentrations.

8.5 RESULTS

8.5.1 Baseline Characteristics

The clinical characteristics of the subjects are shown in Table 8.1. The diabetic subjects were older than the controls (61.1(12.9) vs 41.5(10.8) yrs, p=0.003) but the ratios of smokers to non-smokers and of men to women were not significantly different in the two groups.

Fasting plasma glucose, HbA_{1c}, TG and LDL-Chol concentrations were significantly higher in diabetic subjects than in controls, whereas HDL-Chol was significantly lower in diabetics (Table 8.2). Total-Chol concentration was not significantly different in the groups.

Neither TRAP (801.0(119.9) vs 789.1(106.1)$\mu moll^{-1}$, p=0.823) (Fig 8.2) nor uric acid (0.32(0.09) vs 0.23(0.09)$mmoll^{-1}$, p=0.065) (Fig 8.3) were significantly different in diabetics compared to controls. Uric acid significantly correlated with TRAP (r=0.616, p=0.009) (Fig 8.4) but not with HbA_{1c} (r=0.230, p=0.375).

There was no significant difference between the groups in terms of vitamin E concentration (16.7 (15.4, 23.7) vs 17.9(15.5 21.0)$\mu g.ml^{-1}$, p=0.0.967) (Table 8.2). The vitamin E status was significantly lower in the diabetic group compared to controls (4.81(0.92) vs 5.77(0.92)$\mu M.mM^{-1}$, p=0.032). However, when age was controlled for in an analysis of variance with age as a covariate, diabetes was found not to contribute

Variable	Diabetic Subjects (n = 9)	Non-diabetic Subjects (n = 10)	p value
Glucose ($mmol.l^{-1}$)	10.7(4.2)	5.2(0.5)	0.004
HbA_{1c} (%)	10.1(2.9)	6.4(0.6)	0.005
Total Cholesterol ($mmol.l^{-1}$)	6.9(1.7)	6.0(0.7)	0.164
Total Triglycerides ($mmol.l^{-1}$)	2.4(1.7,3.3)	0.9(0.8,1.7)	0.019
LDL-Cholesterol ($mmol.l^{-1}$)	4.3(0.5)	3.7(0.5)	0.026
HDL-Cholesterol ($mmol.l^{-1}$)	1.1(0.3)	1.7(0.4)	<0.0001
Vitamin E ($\mu g.ml^{-1}$)	16.7(15.4,23.7)	17.9(15.5,21.0)	0.967
Vitamin E status ($\mu M.mM^{-1}$)	4.81(0.92)	5.77(0.92)	0.032

Table 8.2: The comparison of glycaemic control, fasting plasma lipids, Vit E and Vit E status in diabetic and non-diabetic subjects at baseline.

Values are mean (SD) or median (25^{th}, 75^{th} percentile). Comparisons were made using unpaired Student's T-test or Mann-Whitney U-test).

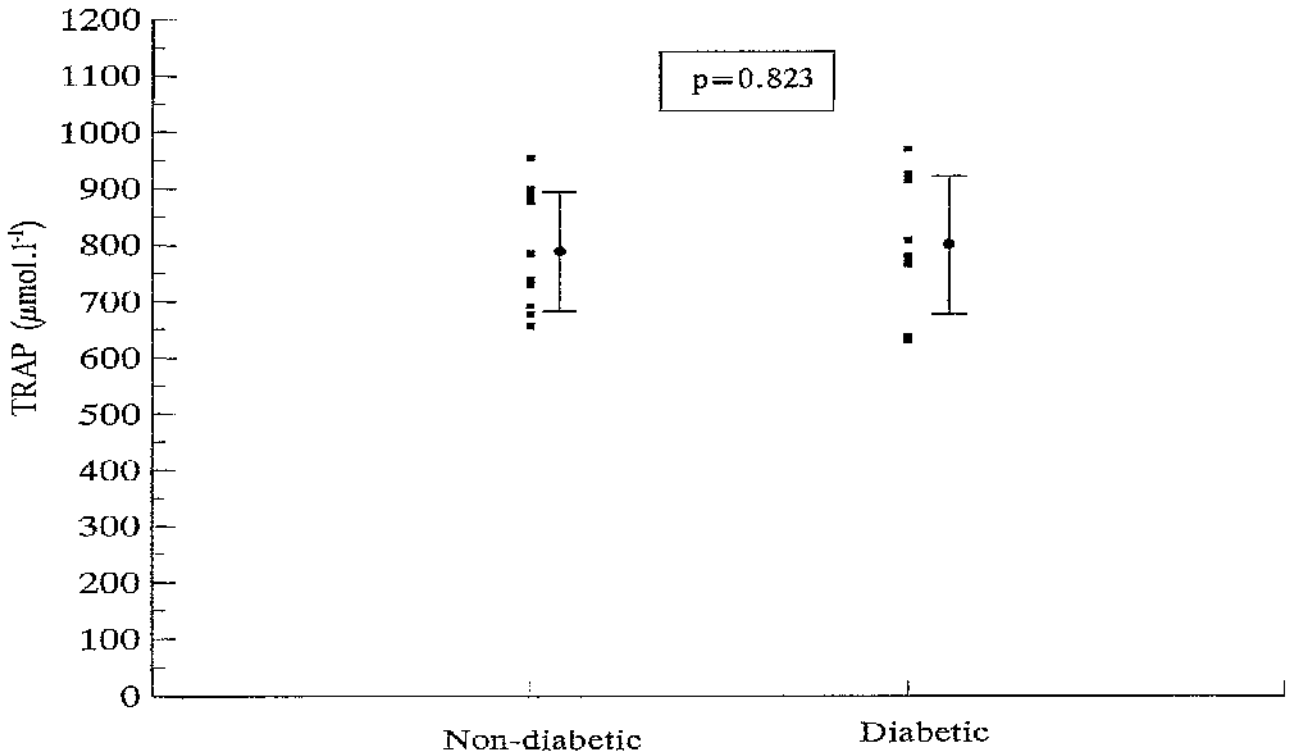


Figure 8.2: TRAP in non-diabetic and diabetic subjects.

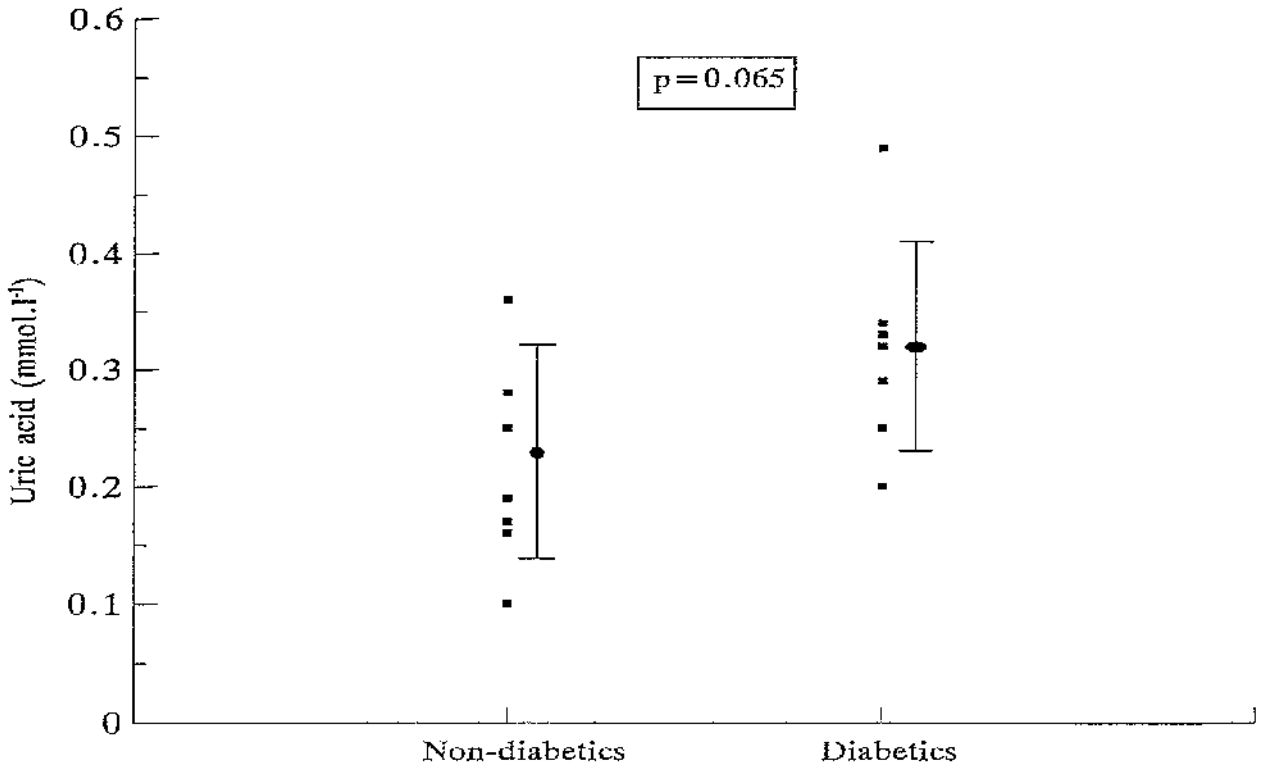


Figure 8.3: Uric acid concentrations in non-diabetic and diabetic subjects at baseline.

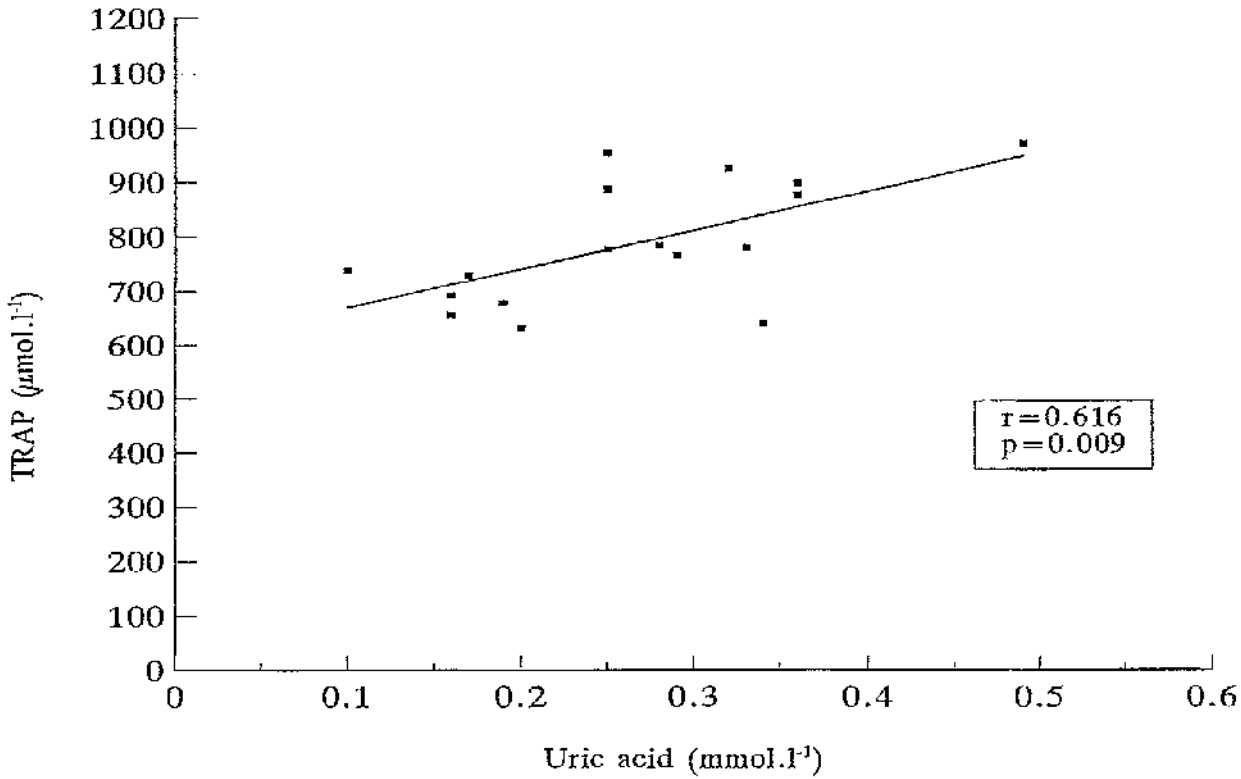


Figure 8.4: The correlation between TRAP and uric acid in all subjects at baseline.

significantly to the variance in vitamin E status ($F=3.036$, $p=0.105$) whereas age did explain a significant proportion of the variance ($F=7.363$, $p=0.018$). The age difference between groups did not alter the conclusions regarding other comparisons at baseline.

TBARS and the duration of lag phase (LAG) of LDL oxidation *in vitro* were not significantly different between the groups. Nor were the other measured aspects of LDL oxidation (Table 8.3).

Considering all subjects together there was no significant correlation between measures of lipid oxidation and glycaemic control (Table 8.4) other than an inverse correlation between the duration of the propagation phase (PROP) and HbA_{1c} and plasma glucose. When diabetic and non-diabetic groups were considered separately this correlation between PROP and glycaemia (HbA_{1c})remained significant for the diabetic subjects ($r_s=0.786$, $p=0.010$) but not the non-diabetics ($r_s=0.316$,$p=0.187$). Also the rate of diene conjugate formation was significantly related to HbA_{1c} and glucose only in diabetics. Vitamin E status did not correlate with TBARS ($r=0.148$, $p=0.546$) (Fig 8.5), nor with LAG ($r=0.065$, $p=0.800$) nor with other measures of LDL oxidation (Table 8.4). TRAP did not correlate with any measure of lipid oxidation (Table 8.4).

In all subjects TBARS correlated significantly with TG concentration (Table 8.5) but not with measures of *in vitro* LDL oxidation. In the separate groups TBARS correlated with TG in non-diabetics ($r_s=0.808$, $p=0.002$) but not in diabetics ($r_s=0.381$, $p=0.156$).

Lipid Oxidation	Diabetic	Non-diabetic	p
TBARS (μmolMDA.l^{-1})	2.0(0.4)	1.9(0.4)	0.878
Duration of Lag Phase (min)	64.3(29.6)	77.5(51.9)	0.511
Duration of Propagation Phase (min)	24.4(22.6,29.7)	28.0(23.2,34.4)	0.286
DC formed per mg LDL (nmoles DC.mg^{-1} LDL)	207.7(26.4)	205.4(39.4)	0.884
Rate of DC formation (nmoles DC.mg^{-1} LDL.min^{-1})	8.3(1.6)	7.5(2.6)	0.436

Table 8.3: The comparison of measures of lipid oxidation between diabetic and control subjects at baseline.

Values are mean (SD) or median (25th, 75th percentile). Comparisons were tested using unpaired Student's t-test or Mann-Whitney U-test. DC refers to total di-ene conjugated species formed *in vitro* during the incubation period.

Lipid oxidation	HbA_{1c}		Glucose		TRAP		Vit E status	
	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	p	Correlation coefficient	p
TBARS (μmolMDA.l^{-1})	0.343*	0.075	0.345*	0.074	0.026	0.915	0.148	0.546
Duration of Lag Phase (min)	-0.304*	0.110	-0.357*	0.073	-0.063	0.8.4	0.065	0.800
Duration of Propagation Phase (min)	-0.435*	0.036	-0.433*	0.036	0.285*	0.126	0.164*	0.257
DC formed per mg LDL (nmoles DC.mg^{-1} LDL)	0.153*	0.272	0.084*	0.371	0.299	0.227	-0.132	0.603
Rate of DC formation (nmoles DC.mg^{-1} LDL.min^{-1})	0.306*	0.101	0.302*	0.104	0.034	0.890	-0.233	0.337

Table 8.4: The correlation between vitamin E status, TRAP, glycaemia and measures of lipid oxidation.

Pearson correlation coefficients are quoted for for normally distributed data.
* Spearman Rank correlation coefficients are quoted for non-normally distributed data.

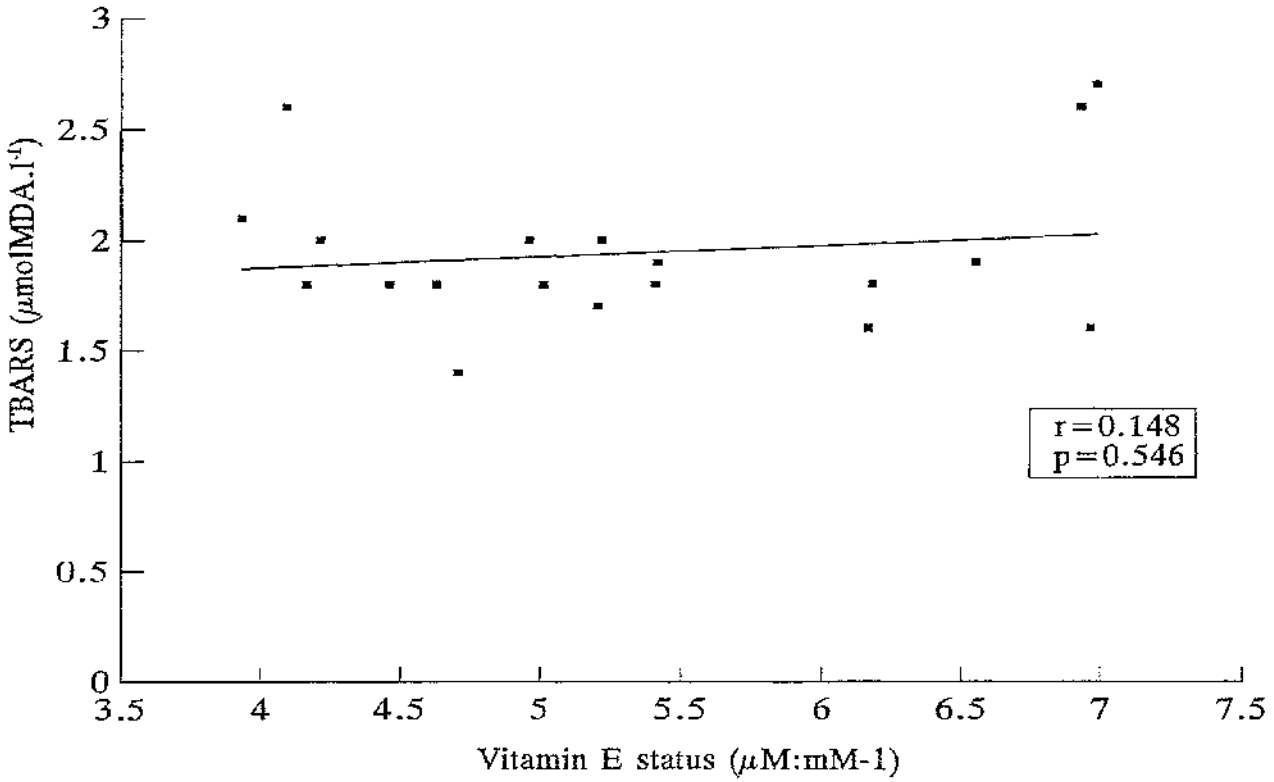


Figure 8.5: The correlation between thiobarbituric acid reactive substances (TBARS) and vitamin E status in all subjects at baseline.

	TBARS (μmolMDA.l^{-1})	
	Correlation coefficient	p value
Triglyceride (mmol.l^{-1})	*0.690	0.001
Total-cholesterol (mmol.l^{-1})	0.163	0.504
LDL-cholesterol (mmol.l^{-1})	0.218	0.369
Duration of lag phase (min)	-0.223	0.375
Duration of propagation phase (min)	*-0.238	0.341
DC formed per mg LDL (nmoles DC.mg^{-1} LDL)	-0.060	0.813
Rate of DC formation (nmoles DC.mg^{-1}LDL.min^{-1})	0.091	0.711

Table 8.5: The correlation of plasma thiobarbituric acid reactive substances (TBARS) with lipids and measures of *in vitro* low density lipoprotein oxidation.

Pearson correlation coefficients are given for normally distributed data and *Spearman rank correlation coefficients for non-normally distributed data.

8.5.2 Effect Of Vitamin C And Vitamin E Supplements

Using analysis of variance for repeated measures, and controlling for the effect of diabetes it was found that there was no significant difference in glycaemic control, plasma lipids (Table 8.6a) or TRAP (Table 8.6b) on the four study days. On the other hand TBARS, uric acid, vitamin E and vitamin E status did significantly differ during the course of the study. Diabetes had no significant influence on these outcomes (Table 8.6b). The rate of diene conjugate formation (DC-RATE) also differed over the course of the study but diabetes did significantly alter this response (Table 8.6 b). No other variables differed significantly throughout the study.

Looking in more detail at those variables which did display significant variance during the study it was found that there was no significant difference in TBARS between visits 1 and 2, that is whilst vitamin C was taken, (1.9(0.4) vs 2.1(0.48)μmol.MDA.l^{-1}, p=0.239). There was a significant fall in TBARS between visits 3 and 4, whilst taking vitamin E (2.3(0.6) vs 2.1(0.3)μmol.MDA.l^{-1}, p=0.049). However, TBARS were significantly higher at visit 3 than at visit 1 (2.3(0.6) vs 1.9(0.4)μmol.MDA.l^{-1}, p=0.015) (Fig 8.6).

Uric acid fell significantly between visit 1 and 2, whilst on vitamin C, (0.27(0.10) vs 0.23(0.09)mmol.l^{-1}, p=0.007) but there was no effect of vitamin E (Fig 8.7). Uric acid fell in diabetic (0.32(0.10) vs 0.27(0.10)mmol.l^{-1}, p=0.026) and in non-diabetic subjects (0.23(0.09) vs 0.20(0.07)mmol.l^{-1}, p=0.07) such that the mean fall in diabetic and non-diabetic was not significantly different (-0.06(0.06) vs -0.03(0.06)mmol.l^{-1}, p=0.318).

Vitamin E fell significantly during the vitamin C treatment period (17.2(15.4,21.2) vs 16.6(13.2,17.9)μg ml^{-1}, p=0.019) and rose significantly during the vitamin E treatment period (17.7(15.5,20.4) vs 24.0(20.0, 26.1)μg.ml^{-1}, p=0.001) (Fig 8.8a). The rise in diabetics was not significantly different from that in non-diabetics.

Vitamin E status fell significantly on vitamin C (5.3(1.0) vs 4.8(0.7)μM.mM^{-1}, p=0.045) and rose on vitamin E (5.4(0.9) vs 7.5(1.6)μM.mM^{-1}, p < 0.0001) (Fig.8.8b). The change in vitamin E status and the change in TBARS during vitamin E administration did not correlate significantly (r_s=-0.058, p=0.406) (Fig 8.9).

The rate of diene conjugate formation (DC-RATE) (Fig 8.10) did not fall significantly between visits 1 and 2, or between visits 3 and 4, but was significantly lower at visit 4 than 1 (5.9(2.0) vs 7.9(2.2)nmol.mg^{-1}.min^{-1}, p=0.011) when the groups were considered together. Diabetes did effect this result (Table 8.6b) in that when the groups

Variable	Vit C Effect		Vit E Effect		Overall treatment effect		Effect of diabetes on treatment effect	
	Visit 1	Visit 2	Visit 3	Visit 4	F_d	p	F_d	p
Fasting glucose ($mmol.l^{-1}$)	7.8(4.0)	7.5(4.0)	7.3(3.1)	7.1(3.8)	0.99	0.404	0.21	0.889
HbA_{1c} (%)	8.2(2.8)	8.0(2.7)	8.0(2.7)	8.4(3.0)	0.79	0.513	0.21	0.886
Total-cholesterol ($mmol.l^{-1}$)	6.4(1.3)	6.3(1.2)	6.4(1.2)	6.2(1.2)	0.93	0.433	0.86	0.469
Triglyceride ($mmol.l^{-1}$)	1.6(0.8,3.0)	1.7(0.8,2.6)	1.4(0.9,3.1)	1.4(0.9,2.1)	1.28	0.291	1.45	0.239
LDL-cholesterol ($mmol.l^{-1}$)	4.0(0.6)	4.0(0.9)	4.1(1.0)	4.0(1.1)	0.09	0.965	0.55	0.648
HDL-cholesterol ($mmol.l^{-1}$)	1.4(0.5)	1.4(0.4)	1.5(0.4)	1.5(0.4)	0.61	0.613	2.06	0.119

Table 8.6a: The effect of vitamin C, and E, supplementation and the interaction between supplementation and diabetes on measures of glycaemic control and lipids.

Values are mean (SD) or median (25^{th},75^{th} percentile). F and F_d are variance ratios computed by *Manova for repeated measures analysis*. F is the ratio of variance attributed to treatment, F_d the ratio attributed to the presence of diabetes.

Variable	Vit C Effect		Vit E Effect		Overall treatment effect		Effect of diabetes on treatment effect	
	Visit 1	Visit 2	Visit 3	Visit 4	F	p	F_d	p
TBARS	1.9(0.4)	2.1(0.5)	2.3(0.6)	2.1(0.3)	4.13	0.011	0.82	0.490
LAG	71.6(42.8)	70.0(42.7)	84.3(35.6)	85.3(23.5)	1.03	0.393	1.95	0.138
PROP	26.2 (23.1,31.1)	24.8 (21.0,32.9)	30.4 (27.0,31.8)	29.1 (22.9,31.9)	0.77	0.519	2.29	0.096
DC-CONC	206.4(33.3)	195.0(35.5)	180.6(39.9)	165.2(37.8)	2.37	0.091	0.94	0.432
DC-RATE	7.9(2.2)	7.7(2.1)	6.0(1.6)	5.9(2.0)	4.89	0.008	2.99	0.049
TRAP	794.7(109.8)	814.9(156.7)	825.7(179.8)	885.1(165.4)	1.75	0.185	0.16	0.908
Uric Acid	0.27(0.10)	0.23(0.09)	0.29(0.11)	0.30(0.10)	8.18	0.000	0.09	0.967
Vitamin E	17.2 (15.4,21.2)	16.6 (13.2,17.9)	17.7 (15.3,20.4)	24.6 (20.0,26.1)	8.95	0.000	0.02	0.997
Vitamin E status	5.3(1.0)	4.8(0.7)	5.4(0.9)	7.5(1.6)	25.4	0.000	0.57	0.638

Table 8.6b: The effect of vitamin C, and E, supplementation, and the interaction between supplementation and diabetes, on measures of lipid oxidation and plasma antioxidants.

Values are mean (SD) or median (25^{th},75^{th} percentile). F and F_d are variance ratios computed by *Manova for repeated measures analysis*.
F is the ratio of variance attributed to treatment, F_d the ratio attributed to diabetes. LAG is the duration of the lag phase of *in vitro* LDL oxidation, PROP is the duration of the propagation phase, DC-CONC is the total diene conjugated species formed per mg LDL, and DC-RATE is the DC-CONC per minute.

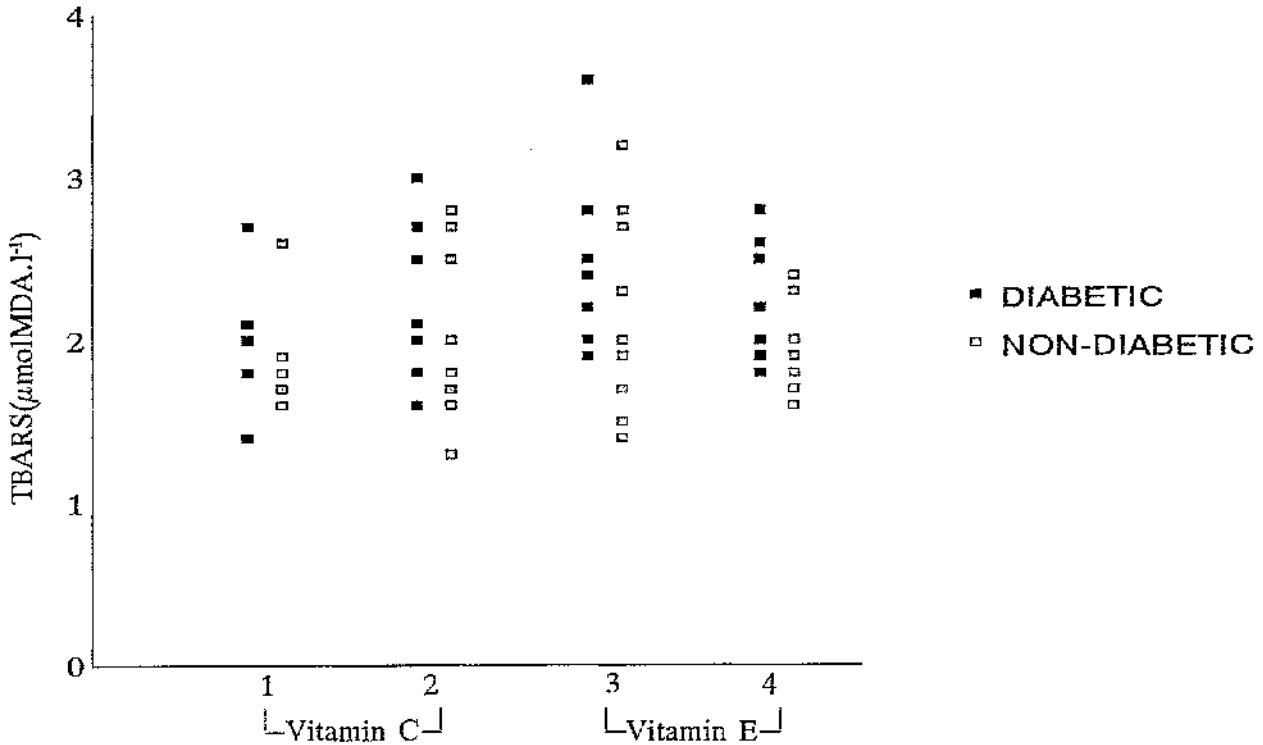


Figure 8.6: The effect of vitamin C and vitamin E supplements on TBARS in diabetic and non-diabetic subjects.

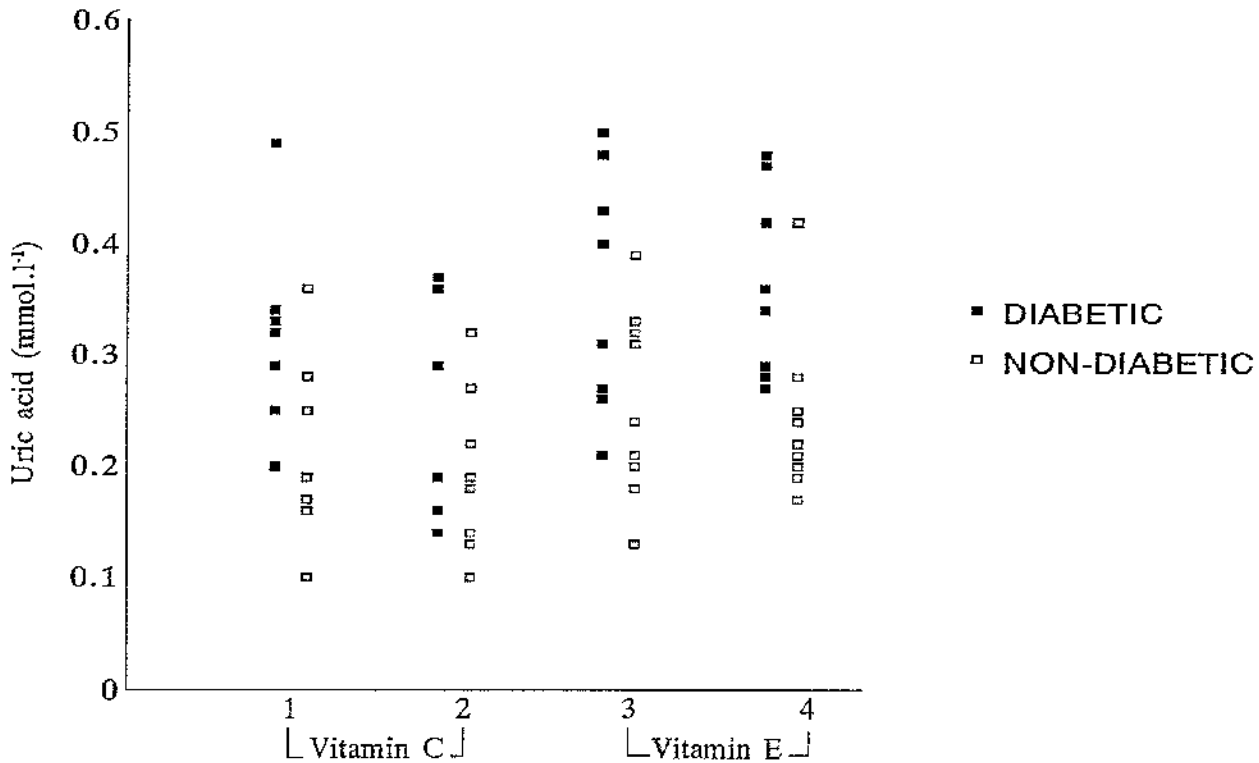


Figure 8.7: The effect of vitamin C and vitamin E supplements on uric acid concentration in diabetic and non-diabetic subjects.

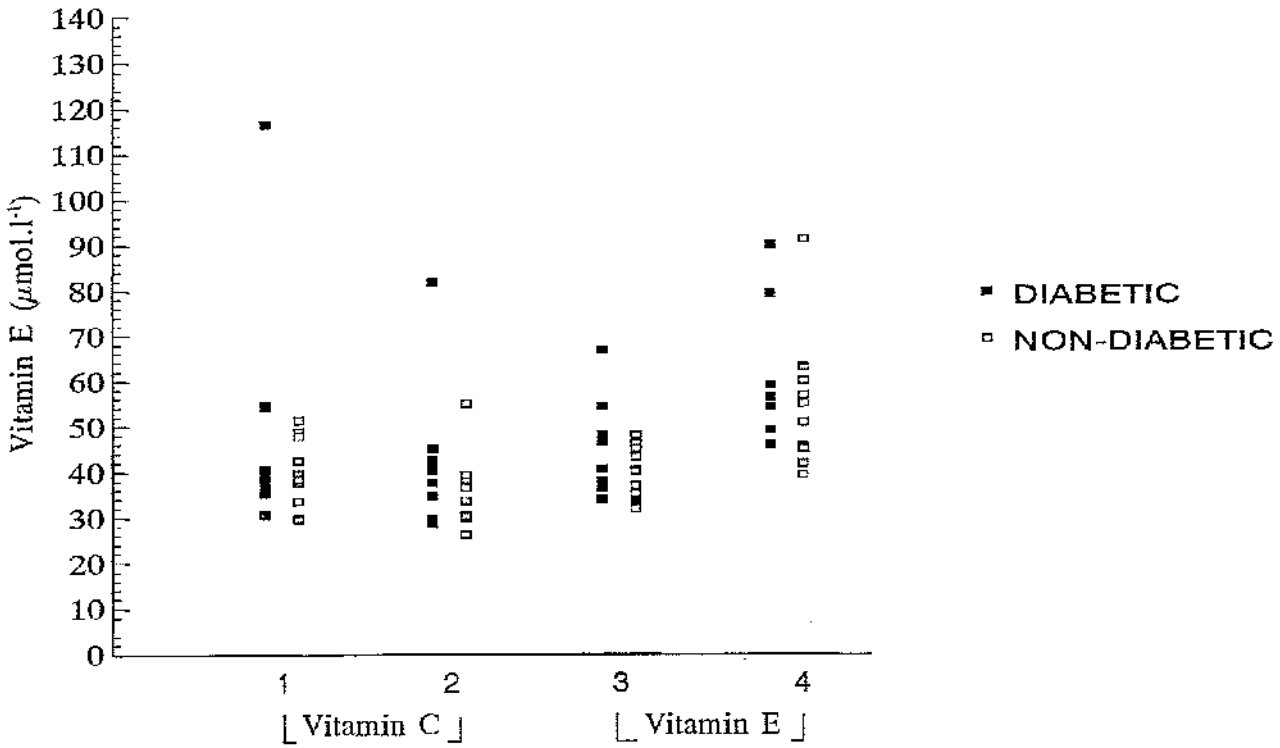


Figure 8.8a: The effect of vitamin C and vitamin E supplements on vitamin E concentration in diabetic and non-diabetic subjects.

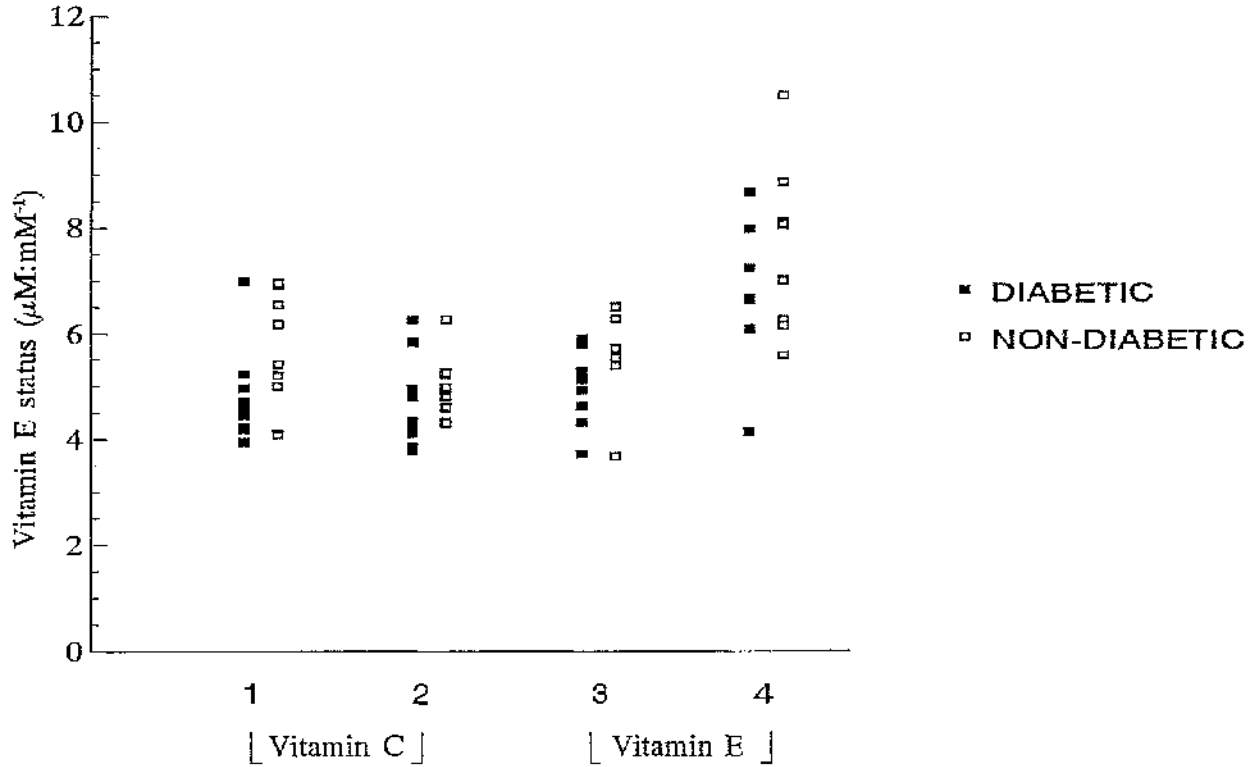


Figure 8.8b: The effect of vitamin C and vitamin E on vitamin E status in diabetic and non-diabetic subjects.

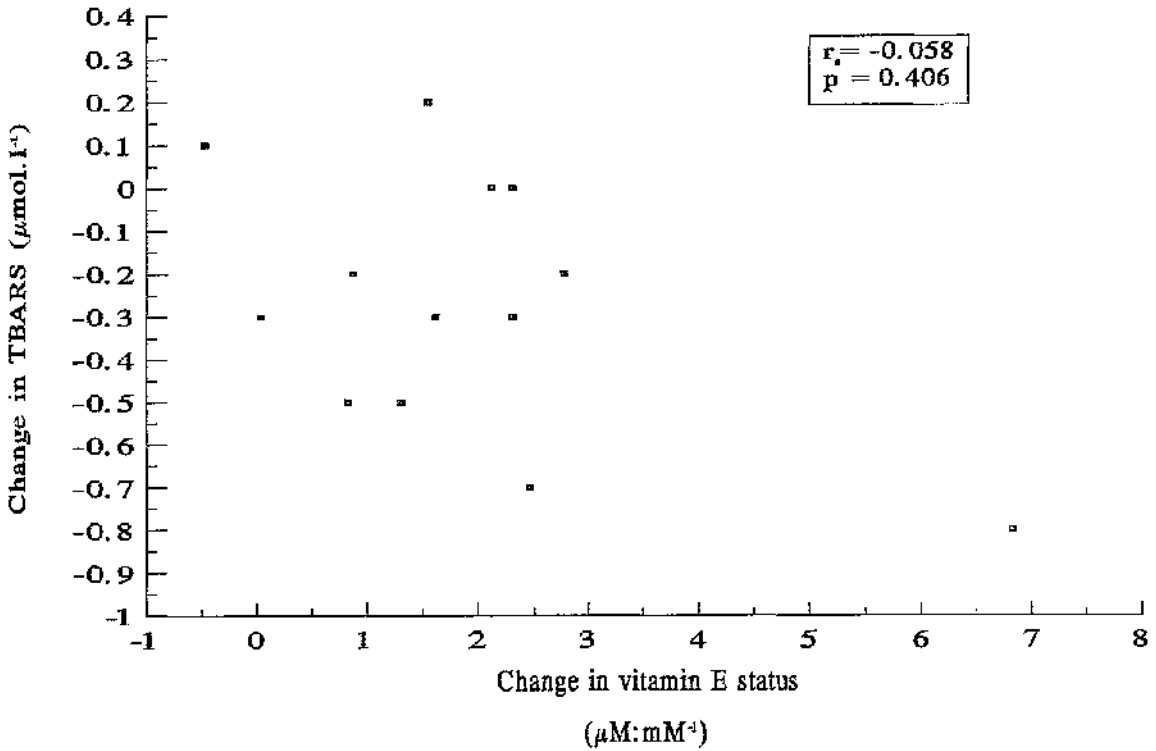


Figure 8.9: The correlation between the change in vitaminE status and the change in thiobarbituric acid reactive substances.

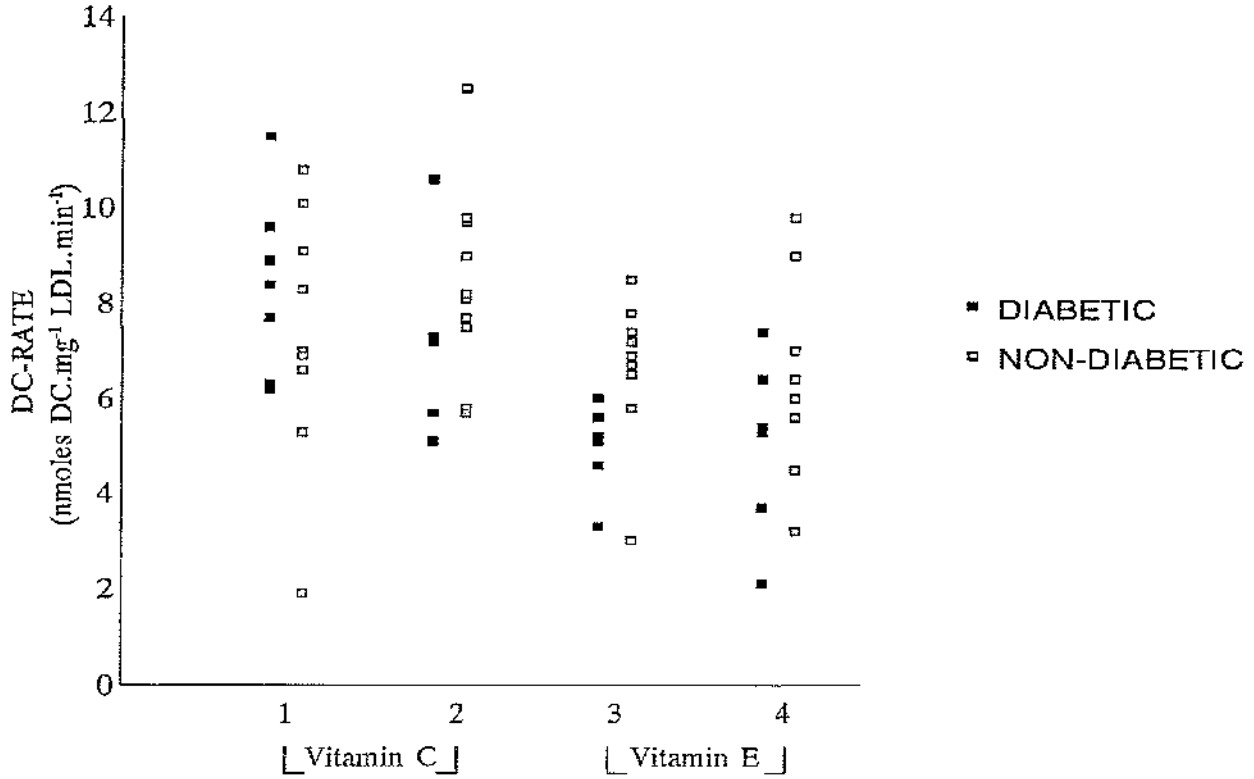


Figure 8.10: The effect of vitamin c and vitamin supplements on the rate of diene conjugate formatio (DC-RATE) during *in vitro* oxidation of low density lipoprotein.

were considered separately the DC-RATE was significantly lower at visit 4 than at 1 in diabetics (8.3(1.6) vs 5.1(1.7)nmol.mg^{-1}.min^{-1}, p=0.002) but not in non-diabetics (7.5(2.6) vs 6.5(2.1)nmol.mg^{-1}.min^{-1}, p=0.381).

8.6 DISCUSSION

This study was designed to act as a pilot for a study the principle aim of which would be to assess the effects of vitamin C and E on antioxidant levels and lipid peroxidation and which would secondarily allow the response be compared in diabetics and non-diabetics. In this pilot study since the subjects act as their own controls in the design used it is not critical, in terms of the main analysis of treatment effect, that the diabetic and non-diabetic subjects differed in age and presence of vascular complications. However these differences could confound effects interpreted as due to diabetes. I will therefore concentrate the discussion on the treatment effect but because of the pilot nature of the study and because age and the presence of vascular complications in the groups would be expected to operate, if anything, in the same direction as diabetes, exaggerating rather than masking differences between the groups, I have analysed differences between groups at baseline and in terms of response to vitamin supplementation, looking for trends that might guide future studies and to allow statistical power calculations be performed. I did control for age statistically but doing so altered only the conclusions reached regarding the comparison of vitamin E status between diabetic and non-diabetic subjects at baseline and did not alter the significance of the effects of vitamin supplementation therefore I have presented the statistics elsewhere without controlling for age. In the definitive study the effect of diabetes on baseline characteristics and upon treatment effect would be assessed by comparing groups matched for vascular disease and age.

8.6.1 Comparison Of Groups At Baseline.

The differences observed at baseline in plasma glucose, HbA_{1c}, and fasting lipids between diabetic and non-diabetic subjects were as expected and as frequently reported before (82). I found no significant difference in TRAP between the groups at baseline in agreement with results reported in chapter five suggesting again that diabetes *per se* is not asociated with alterations in overall plasma antioxidant status. However the lack

of difference may be due to the power of the study since the power to detect a difference of 10% at the 0.05 level of significance with ten subjects in each group would only be 31%. To detect such a difference with a power of 80% would require approximately 30 subjects in each group (215, 300) and the differences actually observed are much smaller than 10% suggesting that even many more subjects than 30 might need to be studied. This requires to be taken into account in future studies.

Uric acid concentrations were not significantly different in the diabetic and non-diabetic subjects studied. This does not necessarily mean that diabetes has no effect on uric acid but may reflect the balance between two opposing effects of diabetes. These subjects had macrovascular disease and were hypertriglyceridaemic and, although it was not measured they were also very likely to be insulin resistant (76). These features have been associated with hyperuricaemia (297, 298) and so hyperuricaemia might have been anticipated in this group. However, it may be that when hyperglycaemia coexists it has an opposing effect, lowering uric acid levels by impairing uric acid reabsorption in the kidney, two such opposing influences would result in no overall difference in uric acid levels in diabetes as seen here. Lack of statistical power could also explain the insignificant result found, although power calculations suggest that 17 subjects in each group would be sufficient to detect the difference that existed with a power of 80% at the 0.05 level. Uric acid concentrations correlated significantly with TRAP, consistent with the observation that it is a major contributor to the value of TRAP (26). In view of its role as an antioxidant (29), its reported abnormality in diabetes (294-296) and its association with cardiovascular risk factors (202, 298), closer evaluation of uric acid may be worthwhile.

Vitamin E concentrations were not significantly different comparing diabetic and non-diabetic subjects whereas vitamin E status was significantly lower in diabetic subjects than in controls. Hence correcting for plasma lipids revealed that this group of diabetics may have lipids relatively less well protected from oxidation. However when age was statistically controlled for diabetes was found not to significantly influence vitamin E status and the difference between the groups to be significantly related to their age difference. This effect of age is in keeping with the results of chapter seven where it was discussed more fully in Section 7.6. The effect of diabetes on vitamin E status will require final clarification in age matched comparisons but both of these studies would suggest that diabetes *per se* is not associated with alteration in vitamin E status. Power calculations suggest that 17 subjects in each group would allow detection of a significant result at the 0.05 level with a power of 80%. Vitamin E status did not

correlate with any *in vitro* measure of lipid oxidation or with TBARS and is in keeping with the impression that factors other than vitamin E content are involved in determining the resistance of lipids to oxidation. Also, TRAP and uric acid concentrations did not correlate with TBARS which may again suggest that antioxidants are not a major determinant of lipid oxidation *in vivo* or it could reflect the problems of using TBARS as a marker of oxidation since TBARS concentrations are determined by removal as well as production and TBARS may arise by mechanisms other than FR attack on lipids and all of this in addition to the lack of specificity of the TBARS assay. No measure of lipid oxidation, including TBARS, was found to be significantly different in the two groups at baseline. This is not consistent with the idea that more lipid peroxidation occurs in diabetes or individuals with macrovascular disease (67, 271). Nor is it consistent with my finding in the previous study that TBARS were higher in diabetics and suggests that in the case of TBARS this may be due to lack of power and indeed from this study I would calculate that to detect a 10% difference in TBARS at the 0.05 level with 80% power groups of 64 would have to be examined. The difference in the mean LAG between diabetic and non-diabetic subjects of 13 minutes suggests that 150 subjects would have to be studied to detect such a difference at the 0.05 level with a power of 80%. Although measures of oxidation did not differ between diabetics and non-diabetics the duration of the propagation phase (PROP) inversely correlated with HbA_{1c} and fasting plasma glucose. The relationship was strongest in the diabetic group but this may simply reflect the wider distribution of HbA_{1c} and glucose in the diabetic subjects. Since DC-RATE is derived from PROP it was not surprising that DC-RATE was found to positively correlate with measures of glycaemia. However the extent of LDL oxidation ($DC.mg^{-1}$) did not correlate with glycaemia. These results raise the possibility that glucose increases the rate of LDL peroxidation but not its extent. Considering that when the experiment is performed, LDL is in fact isolated from plasma it cannot be hypothesised that glucose *per se* is having this effect since glucose is no longer present but rather that glucose may modify LDL and then that modified LDL peroxides more rapidly. This idea would be consistent with the suggestion that glycation and oxidation may be associated (74, 102). *In vivo*, if more rapid peroxidation does occur in association with higher glucose levels, but removal of peroxidised material is unaltered, then higher steady state levels of peroxidised material would be expected and this might help explain the higher TBARS reported by others in diabetic subjects (65-69). In addition if direct toxicity of lipid peroxides is an important factor in atherogenesis then increased lipid peroxide

production rate could be a mechanism whereby hyperglycaemia contributes to atherogenesis. In this study despite the correlation between DC-RATE and glucose concentration, I did not find that TBARS concentration, a measure of lipid peroxidation *in vivo*, was greater in diabetics than controls nor did TBARS correlate with glycaemia and, although this could well be explained by the power of the study, it raises concern about the source of TBARS. If TBARS are derived from LDL oxidation it might be expected that those factors which influence LDL oxidation should influence TBARs concentration, this was not the case for glucose concentrations. Also, if TBARS do derive from LDL oxidation then some relationship between measures of *in vitro* oxidation of LDL and TBARS might have been expected but was not found. The fact that TBARS concentration was significantly related to TG concentration would be more in keeping with the possibility that they are derived from triglyceride rich lipoproteins such as VLDL as opposed to LDL. In the study in chapter seven TBARs also correlated with TG, although when age was controlled for this correlation was lost, nevertheless reasons why TBARs might correlate with TG and the significance of such a correlation was discussed in section 7.6.

8.6.2 The Effect Of Vitamin Supplementation

In agreement with other studies I found supplementation of vitamin C or E had no effect, in particular no adverse effect, on glycaemia or lipid concentrations in diabetics or non-diabetics (120, 194, 282, 302). However it has also previously been reported that although vitamin C (303) and vitamin E (103) were without effect on plasma glucose they nevertheless reduced glycation of haemoglobin and plasma proteins. The lack of effect on HbA_{1c} in my study may have several reasons. Davie *et al* administered vitamin C for 3 months and Ceriello *et al* administered vitamin E for 2 months whereas supplementation in this study was for 3 weeks. Duration of treatment may be important *per se* but is perhaps made more so by the relatively long half-life of haemoglobin in plasma which more nearly approaches 3 months than 3 weeks. Also as suggested by Davie *et al*, the method of measuring haemoglobin glycation may be relevant. Complexes of vitamin C with haemoglobin may be detected by agar gel electrophesis as glycated haemoglobin, masking any fall in true glycosylated species. It may be more appropriate to use affinity chromatography techniques in future studies to determine true glycosylation. Glycosylation may be relevant pathophysiologically via free radical (18, 131, 288), and non free radical (85, 86), mechanisms and

consequently factors affecting it may be important and worthy of further investigation. As others have reported large alterations in plasma vitamin C using similar doses to those employed in this study I assumed a rise in vitamin C would occur and did not measure plasma vitamin C levels *per se* but anticipated a rise in TRAP levels because vitamin C has been reported to contribute up to 24% of TRAP (26). However I observed no change in TRAP during vitamin C supplementation. Although statistical power must again be considered, other explanations also exist. There is little reason to suspect that vitamin C was not absorbed, one possibility which then arises isthat other contributors to TRAP fall in proportion to the rise in vitamin C. This is born out by this study in that uric acid, and vitamin E concentrations fall during vitamin C supplementation and uric acid is a major contributor to TRAP (35-65%) and vitamin E a minor one (5-10%) (26). There are several possible explanations for these apparent falls in uric acid and vitamin E. Vitamin C is a urocosuric agent inhibiting the reabsorption of filtered/secreted uric acid in the renal tubules hence increasing its excretion (290, 296), and possibly inducing a fall in plasma levels. Vitamin C can also simply interfere with the assay for uric acid (304) falsely suggesting lower levels of uric acid and although this would help explain a low measured concentration of uric acid during vitamin C supplementation it would not explain the failure of TRAP to increase. A simple modification of the uric acid assay can eliminate the possibility of interference (304) and should be adopted in future studies. Neither of these problems are known to arise with the assay of vitamin E but a third possible reason why uric acid falls on vitamin C might also explain the fall in vitamin E. In certain circumstances vitamin C can act as a pro-oxidant rather than an antioxidant (252, 305). Uric acid and vitamin E could then be consumed in the defence against this pro-oxidant state especially as they are both involved in recycling reactions with vitamin C (29).

The pro-oxidant effect of vitamin C is based upon the fact that it can oxidise in the presence of transition metal ions, especially Fe^{3+} (252, 276). In the process Fe^{3+} is reduced to Fe^{2+} which, as previously described, is capable of reacting with lipid hydroperoxides and O_2 to generate free radicals (FR)(Section 1.2.1). In fact the proxidant effect of vitamin C may depend upon the presence of all three components, ascorbate, transition metals and hydroperoxides, and may explain the finding that EDTA abolished the ascorbate enhanced hydroperoxide-dependent peroxidation of rat liver microsomes (305). Presumably as a consequence of such reactions mixtures of vitamin C and metal ions can actually be employed experimentally as FR generating systems (13, 306). Evidence of the pro-oxidant effect of such combinations is provided by

findings such as enhanced lipid peroxidation and FR damage in isolated rat nuclei in the presence of ascorbate and copper or iron (306). It is argued that *in vivo* a very careful balance of ascorbic acid, transition metal ion availability and metal chelators can maintain the equilibrium in favour of an antioxidant effect for vitamin C (252). *In vitro*, by increasing the concentration of only vitamin C in human plasma, it has been shown that the antioxidant activity of vitamin C is concentration dependent falling as concentration rises however in these circumstances a frankly pro-oxidant effect was not observed (261). Administering vitamin C to an intact animal on a normal diet as in this study is, however, not the equivalent of *in vitro* loading of plasma. Ingestion of vitamin C is known to increase Fe^{2+} absorption from the gut (307, 308). Hence ingestion of vitamin C inevitable means that more Fe^{2+} is also absorbed. This may interfere with the balance mentioned above and shift the equilibrium in favour of oxidation.

The inability to separate the absorption of Fe^{2+} from that of vitamin C during vitamin C supplementation may explain several reported anomalies. Despite the sparing effect of vitamin C on vitamin E *in vitro*,vitamin C fed to guinea pigs did not alter the turnover of vitamin E (309) also vitamin C did not reduce the elevated levels of lipid peroxidation products (malondialdehyde and diene conjugates) seen in streptozotocin diabetic rats (310). However the latter group did report a rise in vitamin E levels on vitamin C a discrepancy with my results which I cannot explain other than in possible species differences. The finding that the absolute levels of the oxidised form of vitamin C (dehydroascorbate) increase with oral supplementation in human controls and non-insulin dependent diabetics is in keeping with the possibility that more unwanted oxidation of vitamin C occurs as oral intake is increased (119, 120). Another related mechanism whereby vitamin C could enhance FR catalysed reactions is suggested by the finding that ascorbate can inhibit the ferroxidase activity of caeruloplasmin and so inhibit the oxidation of Fe^{2+} to Fe^{3+} (311).

TBARS concentrations changed throughout the course of this study but the pattern of change was rather odd. They rose during vitamin C supplementation and continued to rise after vitamin C supplements had stopped being significantly higher than baseline only at visit 3. They then fell significantly between visits 3 and 4 when vitamin E was taken. Several explanations could be afforded for this pattern of change but again it would be consistent with increased oxidative stress during vitamin C supplementation although one would also have to postulate a delayed effect of vitamin C which was not anticipated as vitamin C is water soluble and not stored to any extent. It is not clear

how quickly vitamin C levels return to "normal" when supplementation stops but Davie *et al* did show that 4 weeks after cessation vitamin C concentrations had returned to pretreatment levels (303) and if the uric acid effect is due to vitamin C it had resolved by visit 3, three weeks after cessation. However the effect on TBARS need not be as direct as upon uric acid and display more of a delay. This could be clarified in future studies by following vitamin C concentrations and allowing a longer washout after the vitamin C phase. Designing the study such that some individuals receive vitamin C followed by E, and others E followed by C, would also allow statistical verification that no delayed effect existed or interactions between vitamin C and E occurred (312). The fall in TBARS which occurred between visits 3 and 4, whilst vitamin E was being taken, could be due to withdrawal of a possible oxidative effect of vitamin C or be due to the antioxidant effect of vitamin E. A correlation between the change in vitamin E status and fall in TBARS would have supported the latter but did not exist. Another possible explanation for the TBARS changes is that TBARs concentrations fluctuate significantly simply with time and had nothing to do with the trial, this possibility could be excluded in future studies by performing more than one baseline assessment during a prolonged run in period. The samples pre and post vitamin supplements were assayed in a single assay so that the observations are not a batch-effect.

The suggestion of a pro-oxidant, and so possibly, harmful role for vitamin C seems to be at odds with accumulating epidemiological evidence of a negative association between plasma concentrations of vitamin C and risk of atherosclerotic disease morbidity and mortality (198, 199, 279). However from these epidemiological studies the estimated intake of vitamin C required to achieve maximum prophylactic effect against health hazards including vascular disease was of the order of 100mg daily (198) not the 1000mg pharmacological doses used in this and other studies. It is possible that once optimum levels are exceded adverse effects arise. Apart from possible pro-oxidant effects of high dose vitamin C other adverse effects such as the formation of oxalate kidney stones (313) would need to be considered if ever it were planned to advise populations to increase their vitamin C intake substantially. What the optimum dose is has yet to be established.

Of the measures of copper stimulated oxidation of LDL *in vitro*, only the rate of DC formation in the propagation phase varied significantly during the study but the significant difference did not occur in association with either vitamin C or E supplementation but during the intervening period making it difficult to explain and questioning its significance. A reduction in the rate of peroxidation is also not

consistent with the rise in plasma TBARS, although this conclusion presumes that a similar slowing of peroxidation occurs *in vivo,* and that TBARS are a reflection of LDL oxidation neither of which is proven to be so. Also if the reduction of DC-RATE has got anything to do with vitamin C administration it is inconsistent with current understanding of how vitamin C operates to reduce LDL oxidation. Although vitamin C has been shown to increase the duration of the lag phase of lipid oxidation in plasma and isolated LDL when added to the medium *in vitro* it is believed to do so by intercepting radicals in the medium or by regenerating tocopherol, whilst remaining in the aqueous compartment (275, 276). Vitamin C does not appear to directly associate with LDL or alter its composition *per se*, so that when LDL is isolated from its plasma, as it is in this study, it is isolated from any effect of ingested ascorbate therefore it would not be expected that vitamin C be associated with alterations in LDL oxidation *in vitro*. This may simply be a false positive result. Even if it were not, the pathophysiological relevance of alterations in the rate of LDL oxidation *in vitro* is theoretical and untested currently as discussed earlier (Section 8.1.1).

In addition to the fall in plasma vitamin E concentration and vitamin E status during vitamin C supplementation, as already discussed, they both rose significantly during the vitamin E stage. This effect was unaffected by the presence or absence of diabetes and the rise observed in diabetic and non-diabetic subjects was not significantly different suggesting that vitamin E is absorbed and handled similarly in both groups. Despite this rise of approximately 36% in vitamin E concentrations there was no evidence of reduced lipid oxidation either *in vivo* (TBARS) or *in vitro*. This is in disagreement with studies in which LDL was enriched by either exposure to vitamin E /ethanol mixtures *in vitro* (28, 46, 144, 289) or by oral ingestion of vitamin E prior to LDL isolation (28, 46). In both types of study vitamin E enriched LDL was found to have prolonged LAG. The apparently contradictory results from this study may be explained in several ways. I used all-racemic α-tocopherol acetate (all-rac α-TOH) which is a synthetic mixture of the eight stereoisomers of the naturally occur RRR isomer of α-tocopherol (RRR α-TOH) (219) which was the supplement used in other studies. However this difference is unlikely to explain the contradictory results because the antioxidant activity of these isomers are little different. Taking the antioxidant activity of all-rac α-TOH as 100% it was reported that the activity of RRR α-TOH was 110% (314). However the bioavailability of all-rac α-TOH is less than that of RRR α-TOH (315) and I also used a smaller dose of vitamin E than was used in the other studies quoted. The result of these two facts was that supplementing with 300mg (300IU) in this study resulted in

a 36% increase in plasma vitamin E concentration. This may simply not be sufficient to bring about a change in LDL oxidation since a doubling of vitamin E content of LDL was seen to increase by only 30% the dose of γ-irradiation required to induce a standard amount of peroxidation (144). Also a 2.5 fold enrichment of LDL with vitamin E was accompanied by a prolongation of the LAG of copper stimulated LDL oxidation from 4-6 hours by 2 hours, in other words a doubling of LDL content of vitamin E produced only a 30-50% rise in LAG (46) and to achieve this effect subjects ingested 1.45g of RRR α-TOH. Hence very large doses of vitamin E may be required to enrich LDL enough to induce detectable changes in its resistance to oxidation. The enrichment achieved in this study was less than in the other studies quoted and may be the most important reason for the different results found. However studying the *in vitro* enrichment of LDL from a single donor Cominacini *et al* found a very close relationship between the increase in vitamin E content of LDL and duration of the lag phase, and that only small increments in vitamin E content were required to induce large changes in LAG. They also found that increasing the vitamin E content was significantly inversely related to the rate of propagation (289). An explanation for these different observations may be the individual variation which has been found to exist. Esterbauer *et al* studied the effect of doses of vitamin E between 150IU and 1200IU and reported that there was marked individual variation in the ability of vitamin E to protect LDL from oxidation and in some individuals vitamin E had no significant effect (316) and so subject differences between my study and those of others may have contributed to differences in results. Subject heterogeneity and the size of doses of vitamin E required to alter LDL oxidation suggests again that factors other than vitamin E are involved in determining LDL's susceptibility to oxidation.

If large doses of vitamin E are required to alter oxidation of LDL it brings in to question whether or not this can be the mechanism whereby vitamin E appears epidemiologically to afford protection from vascular disease. Again from epidemiological studies the intake of vitamin E which would appear to provide prophylaxis is in the region of 60IU (198) much less than the dose I have studied and very much less than those shown to reduce LDL oxidation. Perhaps vitamin E works to protect against vascular disease morbidity and mortality via one of the other mechanisms discussed in chapter seven (Section 7.1.2).

The safety of large doses of vitamin E also remains to be established, 300mg per day is pharmacological and a dose of this order (400mg all-rac α-TOH) was observed to modify eicosanoid metabolism with reduced platelet thromboxane production in a group

of insulin dependent diabetic subjects (282). This effect may be quite separate from vitamin E's ability to inhibit nonenzymatic FR activity and may relate to a modulation of the enzymes of the arachadonic acid cascade (281). If to observe enhanced LDL resistance to oxidation, doses far beyond those which alter eicosanoid metabolism are required the possibility of far reaching unwanted effects arise. Some, like the reduction of platelet thromboxane in diabetics (282), may be desirable in terms of atherosclerosis but eicosanoids have such wide ranging activities that there are no guarantees that this would be so in all instances. Despite that fear, adverse effects of high doses of vitamin E have not been convincingly demonstrated (302).

The presence or absence of diabetes did not have any significant effect on any of the results discussed above other than on rate of peroxidation in the propagation phase however since I have argued that the significant alteration seen on DC-RATE was most likely to be a statistical error I do not propose to discuss it further. I acknowledge that since diabetic subjects differ from controls in more ways than just the presence of diabetes I cannot be categorical about this negative effect of diabetes and the definitive study in which subjects are matched in terms of vascular disease and age may be necessary but since I anticipated that these differences between the groups would exaggerate rather than mask differences in the variables of interest I suspect that no such differences exist.

In addition to the considerations above, including those of dosage of vitamins used, the power of this study using 10 individuals to detect an effect of vitamin supplements can be assessed and is small. Although the paired nature of the study adds to its power nevertheless looking at LAG during vitamin E supplementation the power of this study to detect 10% alterations at the 0.05 level of significance is only 7% and to detect such differences with a power of 80% would require that approximately 390 subjects would have to be studied (215).

8.7 CONCLUSION

Vitamin C, 1g per day, did not cause a rise in TRAP and in fact was associated with a fall in plasma uric acid concentrations and vitamin E status in both diabetic and non-diabetic subjects. One explanation for these changes is that, at high doses in individuals on normal diets, increases in vitamin C concentration are associated with increases in Fe^{2+} and so with a pro-oxidant as opposed to an antioxidant effect. The increases in

TBARS concentration observed during the trial may reflect a delayed pro-oxidant effect of vitamin C.

Supplementation with 300mg of vitamin E per day was associated with a significant rise in plasma vitamin E concentration and status but did not cause a rise in TRAP, nor did it alter any parameters of *in vitro* oxidation of LDL in a way which suggested that it enhanced resistance of LDL to oxidation. Vitamin E supplementation was associated with a fall in TBARS, a measure of *in vivo* lipid oxidation however these may have been unrelated as the change in vitamin E status did not correlate with the change in TBARS concentration. TBARS concentration did not correlate with any parameter of *in vitro* LDL oxidation raising several possibilities. TBARS concentration *in vivo* may be more influenced by removal than production or are not derived principally from LDL. The correlation of TBARS with TG concentration suggest that they might be derived mainly from the TG rich lipoproteins such as VLDL. Lipid hydroperoxides detected by the TBARS assay also arise *in vivo* during eicosanoid metabolism which can be modified by vitamin E, perhaps the fall in TBARS on vitamin E is mediated by this effect as opposed to an effect on non-enzymatic FR attack on LDL.

The lack of observed effect of these doses of vitamins E and C on the variables measured may be that they were too small, however this raises the question as to whether or not reduced lipid oxidation is the mechanism whereby these vitamins appear in epidemiological studies to protect against atherosclerotic vascular disease morbidity and mortality. This doubt is raised particularly because the doses of these vitamins which epidemiological studies suggest would provide protection are of the order 100mg vitamin C and 60IU vitamin E per day. These conclusions appear to apply similarly to diabetic and non-diabetic subjects but comparison of healthy controls with diabetic subjects without vascular disease, as planned, would have to be performed to verify this impression.

Power calculations on the basis of these results suggest that to detect 10% changes, or differences, in LAG with 80% power at a significance level of 0.05 approximately 400 subjects would have to be studied.

FINAL COMMENTS

Each of the chapters in this thesis describe individual studies which can stand alone and give rise to their own conclusions. These conclusions are discussed in some detail in the relevant chapters and I will not repeat them all again here, rather I would like to outline only the main conclusions, add a few general thoughts and point out again some of the interesting questions raised by the studies performed.

The hypothesis underlying, and unifying, these individual studies is that diabetes is a state in which more free radical oxidation of low density lipoprotein occurs. If low density lipoprotein oxidation is an important event in the genesis of atheroma then it follows that an increase in the potential for low density lipoprotein oxidation in diabetes would help to explain the association of diabetes with atherosclerotic vascular disease. There are theoretically many ways in which free radical oxidation of low density lipoprotein might be influenced by diabetes. In this work I have studied several of these theoretical influences but have found no support for the overall hypothesis.

Many explanations why the individual studies generated negative results have been discussed but one recurring possibility relates to the limitations of the readily available methodologies in the field of free radicals in biology. Also knowledge of free radical processes *in vivo* is currently incomplete, thus the models used to set up more advanced hypothesis may be misleading. I feel advances in methodology and basic knowledge of free radical biology are needed before the questions of their importance in disease will be clearly answered.

The study described in chapter three directly addressed a long standing question reguarding the appropriateness of using diene conjugated fatty acid concentrations as an indirect method of measuring *in vivo* free radical activity. The conclusion was reached that diet has such a large influence on diene conjugated fatty acid concentrations in biological samples that their use as free radical markers must be very limited.

Because questions regarding interpretation of standard methods of studying free radicals exist I have argued a case for and made use of some less publisised techniques however they too raised questions in their interpretation which are discussed in the relavant chapters.

Looking first for a possible source of excess free radical production in diabetes I studied the superoxide anion production by platelets, as platelets are more aggregable in diabetes and are believed to have a role in atherosclerosis. I have to conclude that

platelets do not release superoxide anion. This is not to say that platelets do not release other radicals or species capable of reacting to form free radicals or of taking part in radical reactions. These are all possibilities which could be explored in the future. Also there are many ways, other than *via* free radical processes, by which platelets could influence atherosclerosis.

An assay of the total peroxyl radical trapping activity (TRAP) of plasma was employed to ascertain whether or not antioxidant levels were less in diabetic subjects than in controls. No difference directly attributable to diabetes was observed however the possibility of an interaction between diabetes and smoking which results in reduced antioxidant levels was suggested. Studies with larger numbers of subjects should be undertaken to explore this possible interaction further as such an interaction might help explain the particularly adverse effects of smoking in diabetes.

In diabetic subjects plasma antioxidant levels as represented by TRAP did not correlate with the peroxidisability of plasma lipids as was hypothesised. Whilst bearing in mind the interpretational difficulties generated by the methods used this lack of correlation could also reflect the complexities of both the peroxidation process, as it occurs within lipoprotein particles, and the interaction of water soluble and lipid soluble antioxidants in influencing it. At its simplest it may be that TRAP reflects mainly water soluble antioxidants and that peroxidisability would be more influenced by lipid soluble antioxidants.

However in the studies described in chapters seven and eight I did not demonstrate any association between vitamin E, the major lipid soluble antioxidant, and lowering of lipid oxidation. It was argued that this finding suggested that lipid oxidation is influenced more by factors other than, or in addition to, vitamin E. In a very recent study Reaven *et al* (317) confirmed that the fatty acid composition, lipoprotein density and particle size are also important determinants of susceptibility of low density lipoprotein to oxidation. Also I found that vitamin E status, vitamin E concentration relative to plasma lipid concentration, was not lower in diabetic subjects than in controls although did correlate inversely with age. Hence again it could not be inferred that lower antioxidants in diabetics is a factor in their excess risk of atherosclerotic disease.

It is also salutory to remember that the ease with which lipids oxidise in plasma may not be of relevance anyway. It is possible that oxidation in micro-environments such as in the intima is more important and influenced by yet different factors.

A further study in which vitamin C and vitamin E supplements were taken did not support the hypothesis that these antioxidants might protect lipids from oxidation. In

fact the possibility of a pro-oxidant effect of high dose vitamin C was raised. In addition it was observed that low density lipoprotein was not protected from oxidation *in vitro* by ingestion of doses of vitamin E far in excess of those which epidemiological studies suggest reduce the risk of atherosclerosis. In this study 300mg of vitamin E were taken daily and allowed plasma levels of vitamin E rise by approximately 30% but no significant resistance of lipid to oxidation was seen. In agreement with earlier studies, Reaven *et al* (317) showed that vitamin E enrichment of low density lipoprotein by a factor of between 2-2.5 did increase resistance of isolated low density lipoprotein to oxidaton but this effect was achieved by the ingestion of large, pharmacological, doses of vitamin E (1200mg daily). Such studies suggest that the lower risk of atherosclerosis, in populations ingesting diets marginally richer in vitamin E, is not gained through an effect of vitamin E upon lipoprotein oxidation. One possible alternative is that vitamin E protects by altering eicosanoid metabolism thus prostacyclin/thromboxane activity and platelet function.

The hypothesis that the free radical oxidation of low density lipoprotein is central to the developement of atherosclerosis is intriguing as it allows theoretical explanations for many of the associations observed for this process which is involved in much of the morbidity and premature mortality in the western world. The hypothesis remains unproven but no doubt deserves, and will receive, further exploration.

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