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COMMENTARY

Practical Approaches to Low Density Lipoprotein Oxidation: Whys, Wherefores and Pitfalls*

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The purpose of this review is to bring together the different approaches for studying the oxidation of low density lipoproteins and try to identify some critical factors which will permit greater comparability between laboratories. These issues are discussed both in terms of the variety of exogenous mediators of oxidation applied (transition metal ions, haem proteins, azo initiators, peroxynitrite, cells etc.) and their raisons d'être, as well as the methodologies (formation of conjugated dienes, hydroperoxides, decomposition products of lipid peroxidation, altered surface charge, macrophage uptake) applicable to the different stages of the oxidation and the factors underlying their accurate execution and interpretation.

Keywords: Oxidized LDL, lipid hydroperoxide, conjugated diene, apoprotein B-100

I INTRODUCTION

In recent years there has been increasing interest in the hypothesis that many aspects of atherogenesis, and indeed the thrombosis associated with cardiovascular disease, may be associated with the oxidation of lipoproteins, mainly in the artery wall.[1,2] The corollary of this proposal is that dietary (or supplemented) antioxidants may be protective against cardiovascular disease, for which there is considerable epidemiological evidence. However, it has proved difficult to establish convenient biomarkers in blood, urine or exhaled air for oxidation which would assist in monitoring the effects of antioxidants in short term studies. There have been a number of attempts to measure products of lipid and protein oxidation: the simpler ones, which are often spectrophotometric tests, are questionable because of interference from other plasma factors. The more sophisticated methods are either difficult to perform or require a heavy invest-

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ment in complex equipment which discourages their routine use in large trials. Furthermore, since the oxidation is considered to occur largely in the artery wall, it may not be acceptable to relate measurements in the blood to the arterial location. There have been persistent reports that there are modified low-density lipoproteins (LDL) in the plasma, which are more electronegative than normal LDL, and that they may represent a particle which is more closely related to the modified LDL which may occur in the artery wall.[3]

It is not disputed that the LDL, which may ultimately be oxidised in the subendothelial regions of the artery, is of plasma origin. Since LDL appears to pass readily across the endothelium, modified LDL may migrate back into the plasma.[4] The propensity of the LDL to oxidise may be determined, at least in part, by the properties and composition of the lipoproteins as they are found in the plasma, even though the oxidation may not occur until it has passed into the arterial intima. Therefore, the interest in the effects of oxidation *in vitro* of LDL which has been isolated from plasma seems to have a logical justification. The methodology is founded on the seminal work of Professor H. Esterbauer.[5] There is a need for standardisation of some of the procedures. It is the intention of this article to establish a way forward on this matter and to try to identify some critical factors which will permit greater comparability of the procedures between laboratories. There are already established protocols for some of these methods[6] and the various options for the measurement of lipid oxidation have been reviewed.[6,7] This article is an attempt to revisit some of these points and some new ones.

There is a further use of oxidised lipoproteins in the study of cellular function. It is clear that the requirements for this vary from one set of experimental conditions to another. Nevertheless, it is clear that there is now considerable difficulty in comparing the results appearing in different publications because of the lack of definition in the procedures adopted for the oxidation and, in particular, because of the dearth of detail on the chemical composition of these oxidised preparations.

II ISOLATION OF PLASMA LOW DENSITY LIPOPROTEINS

There are a number of alternative procedures for the preparation of LDL, by far the most common being ultracentrifugation. The classical methods of flotation ultracentrifugation required the sequential stepwise isolation of lipoprotein fractions in increasing order of density, a process sometimes lasting days. A single step method of isolation of LDL has been devised[8] using the rapid vertical rotor technique which claims to produce clean LDL samples. Some laboratory workers find that this does not give a pure sample and that a second (wash) step is used. One of the problems encountered is smearing of VLDL down the side of the tube in the vertical rotor in triglyceride-rich samples. This can be reduced with a near vertical rotor,[6,9] which is available from at least one company. Many operators use the fixed angle rotor for a short spin, which separates the LDL crudely in "vertical rotor" mode, and then reisolate the LDL by a second centrifugation. Good results are obtained using the procedure in Table 1, but others may also be successful. An alternative procedure for use with small samples is to use a table top ultracentrifuge as furnished by Beckman plc which may reach very high speeds and consequently reduce the time required for centrifugation. The single-step method which provides rapidity of isolation thus minimising peroxide formation during isolation, is especially useful for the very small volumes of plasma obtained from patients and protein-contamination has not been found to be a problem in the authors' laboratories. However, if smaller versions of the full scale procedure are used with 5ml centrifuge tubes with the centrifugations reduced to 40 minutes each, excellent results are

TABLE I Procedure for the Isolation of LDL

1. Collect fresh blood into EDTA or ACD (acid-citrate-dextrose) (containing 100μM EDTA) 1:4 dilution. Centrifuge at 3000rpm (800–1000g) at 10°C to separate plasma.
2. Alternatively use plasma frozen with 10% w/v sucrose.[12]
3. Add sodium (or potassium) bromide to adjust plasma density to approximately 1.3g/ml; in order to do this, the volume to be added will be 0.46 × plasma volume.
4. Underlay 15ml of plasma with 0.9% sodium chloride (degassed by boiling for 10mins + cooled prior to use) containing EDTA at final concentration of ~10μM or 2μM DTPA (diethylenetriamine pentaacetic acid) in tubes which are then stopped.
5. Centrifuge at 10°C for 2hour at 170,000g and remove yellow layer of LDL.
6. Put 6ml of 1.15g/ml NaBr density solution [NaBr 195g/L in 100μM EDTA or 2μM DTPA] + 17.5ml NaBr density solution (1.063g/ml in 100μM EDTA) to give a final concentration of ~64μM EDTA and overlay ~15ml LDL.
7. Centrifuge at 10°C for 8–14 hours at 170,000g. The LDL floats near the top of the tubes and is removed.
8. It may be stored at this stage under argon or nitrogen for two weeks in the dark at 4°C. [Argon, being denser than air, is preferable but most laboratories find N_2 perfectly satisfactory for their shorter duration of storage].
9. Dialyse against 5L buffer (vacuum degassed—either 10mM PBS, 100μM EDTA or 12.5mM Tris, 140mM NaCl, 2μM DTPA) over 6h changing the buffer every 30min then in fresh buffer overnight.
10. Filter through 0.22μm sterile filter. If LDL is not being used immediately, omit dialysis and filter before storage under nitrogen at 4°C.
11. Alternatives for rapid preparation of small samples: spin in table top ultracentrifuge 2 × 20min and desalt on a S300 column.

also obtained. In conjunction with the smaller volumes, desalting columns (e.g. Sepharose S300) may be used as a rapid method for eliminating the NaBr. Whichever, centrifugation approach is adopted, ideally the methods should involve isolation of the LDL as rapidly and cleanly as possible:

i) Venous blood should be withdrawn into EDTA so that immediate chelation of any metal ions on the containers or solutions will occur and help to prevent oxidation. Plasma should be separated by low speed centrifugation (800g) for 20 minutes and removed avoiding buffy coat and red cells. At this stage protease inhibitors can be added: the following cocktail has been found to be effective (benzaminidine 0.3% w/v, ε-aminocaproic-n-acid, a serine protease inhibitor at 1.3% w/v, sodium azide 0.05% w/v, gentamicin 0.0005% w/v). Other anticoagulants may be used but EDTA should also be added.

ii) The LDL preparation should be free of other protein contaminants, especially albumin, which may influence the course of the oxidation. This can be checked by SDS/polyacrylamide gel electrophoresis (Fig. 1):[10] agarose gel electrophoresis may not be adequate for this purpose. Isolated LDL may show more than one band on SDS-PAGE because of the proteolytic degradation during isolation. The cleavage products may remain associated with the LDL particle and therefore not be distinguished on the agarose gels.

iii) The LDL should include the major density subfractions (LDL1, LDL2 and LDL3) found in the plasma. It is possible to lose some of the higher or lower density fractions in some of the rapid preparations and these may behave somewhat differently to an oxidative challenge—the highest density component, with the least lipid, is actually the most rapidly oxidised.[11] Equally well, the same can result from the long-spin methods due to alteration of salt gradients during the prolonged centrifugation.

iv) The LDL samples should be fresh as possible so that the oxidative process has not already begun and it should be stored in such a way as to avoid deterioration of the LDL (see below). All reagents should be of the highest grade and have a minimal concentrations of metal ions.

v) All assays should be performed in duplicate at least.

vi) Storage of samples is a considerable problem for population or clinical studies where large numbers of samples are envisaged. LDL solu-

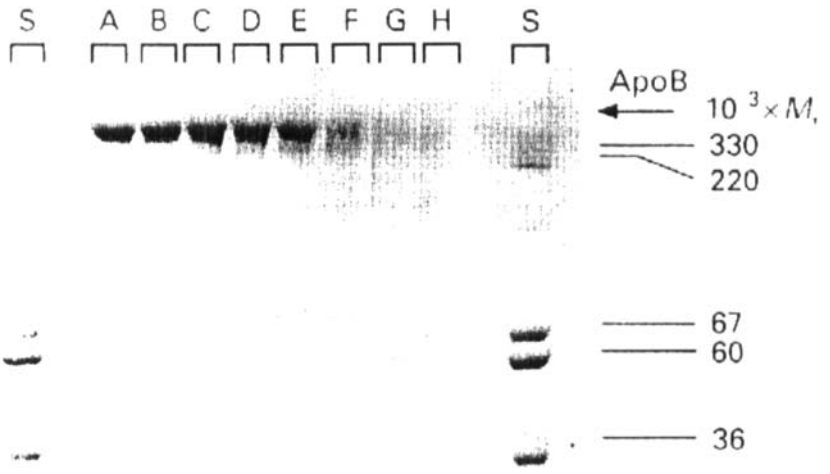

FIGURE 1 SDS-polyacrylamide gel electrophoresis of LDL corresponding to 2mg apoB/ml incubated with 51.2 μmol Cu^{2+}/g of apoB. Lanes A–H, after 0, 2, 4, 6, 8, 12.5, 16.5, 20.5h after initiation of oxidation. M_r markers (Lane S) with values marked to the right [adapted from reference[10]].

tions cannot be frozen without denaturation and frozen plasma samples do not provide satisfactory LDL when thawed. An acceptable alternative for freezing plasma is described by Rumsey et al.[12] in which 10% w/v sucrose is added to the fresh plasma prior to freezing and storage at –70°C. However, when thawed the LDL should be used within a short space of time. Some groups find that they can store their LDL in the presence of 100μM EDTA for considerable periods at 4°C; this is then dialysed to 10μM EDTA prior to use. If such stored LDL is used, it should be checked for the presence of lipid peroxides, consumption of carotenoids (readily observed broadly by eye), for α-tocopherol and for proteolytic fragmentation with SDS-PAGE.

III METHODS FOR INDUCING LDL OXIDATION

1. Introduction

Lipoproteins obtained from plasma are designated as native lipoproteins although there is evidence for the presence of very low concentrations of oxidation products in them. The origin of these products is a matter of debate, whether they are the result of endogenous mild oxidation, of transfer from other tissues or possibly absorbed along with dietary lipids. In a number of disorders, there is increased evidence of the presence of these oxidation products in the plasma lipoproteins, but it is unclear whether the oxidation has occurred in the circulation: this appears to be unlikely. Therefore, oxidised lipoproteins which resemble those isolated from the wall of an atherosclerotic artery have to be simulated by modification *in vitro*. The main problem is that the extent of oxidation can be very variable.

These oxidised forms *in vitro* can be broadly divided into two types. There are those which have been oxidised only slightly ie. minimally-modified LDL,[13] so that some of the lipids have been oxidised (see Section V), but protein modification has not occurred. The others are severely oxidised with extensive protein and lipid modification. Both are valid experimental tools. The minimally modified LDL is also considered to be cytotoxic, but may modulate cellular activity by regulation of the transcription of genes and the translation of protein synthesis. Severely oxidised LDL may represent an end-stage in this process and is profoundly cytotoxic. Since there

is yet no concensus concerning the processes which contribute to the oxidation of the lipoproteins in vivo (there are likely to be several) it is valid to consider a number of agents which may oxidise LDL *in vivo* or simulate compounds which may do so.

Cells of the type present in the arterial wall and found in atherosclerotic lesions, activated macrophages,[14–17] endothelial cells,[17–18] smooth muscle cells,[19] and T-lymphocytes all of which secrete superoxide radicals, hydrogen peroxide and hydrolytic enzymes, have been reported to oxidize LDL (reviewed in ref. 2) but superoxide and hydrogen peroxide generated therefrom are not very reactive *per se* towards polyunsaturated fatty acids. Their reactivity may, in principle, be amplified at lower pH values (through oxidative damage through protonation of superoxide), or in the presence of: (1) available delocalized haem proteins generating ferryl haem protein-derived radicals; (2) transition metal ions generating hydroxyl radical; or (3) nitric oxide, forming peroxynitrite.

Injury to cells and tissues may enhance the toxicity of the active oxygen species by releasing intracellular transition metal ions (such as iron) into the surrounding tissue from storage sites, decompartmentalized haem proteins or metalloproteins by interaction with accessible proteases or oxidants. Such delocalized iron and haem proteins have the capacity to decompose peroxide to peroxyl and alkoxyl radicals, exacerbating the initial lesion.

Oxidation of LDL mediated by agents such as transition metal ions or haem proteins is dependent on the presence of preformed lipid hydroperoxides or minimally modified LDL. In the presence of preformed lipid hydroperoxides, induced by enzymic pathways (lipoxygenase-mediated) or non-enzymic pathways (radical-mediated), propagation of peroxidation can be effected in the vicinity of haem-containing and iron-containing species, generating alkoxyl and peroxyl radicals, which can amplify the damage by initiating further rounds of lipid peroxidation.[20,21]

For studying the resistance of LDL to oxidation a variety of oxidative procedures has been applied. The major approaches (Fig. 2) consist of:

(i) applying a catalyst of the oxidative and reductive decomposition of LOOH ie. agents capable of redox cycling eg. Cu^{++}, haem proteins such as myoglobin or haemoglobin.
(ii) challenging LDL with a free radical species eg. $^{\cdot}OH$, ferryl myoglobin radicals, haemin + hydrogen peroxide, irradiation, peroxyl radical-generating azo initiators, capable of initiating lipid peroxidation.
(iii) cell-mediated oxidative modification which might involve (i) and (ii) or both.
(iv) enzyme-mediated oxidation via lipoxygenase action.

The most commonly used approaches to be described and actively assessed in this review are Cu^{2+}- and haem protein-mediated amplification of LDL oxidation, cell-mediated oxidation and direct initiation by peroxyl radical formation within the LDL molecule, using azo initiators for peroxyl radical generation.

2. LDL Oxidation by Transition Metals

2(i) Cupric Ions

This process is based on the concept that LDL, like other lipoproteins, contain trace amounts of endogenous lipid hydroperoxides which are present in the lipid fractions—largely phospholipid or cholesteryl ester hydroperoxides.[22] When these are exposed to transition metal ions, such as copper, the oxidative and reductive decomposition of the peroxides can occur yielding peroxyl and alkoxyl radical species. This leads to the propagation of radical attack on the polyunsaturated fatty acids and the formation of new lipid peroxides which pass through the same cycle of events until the polyunsaturates are consumed.

$$LOOH + Cu^{2+} \rightarrow LOO^{\bullet} + Cu^{+} + H^{+}$$
$$LOOH + Cu^{+} \rightarrow LO^{\bullet} + Cu^{2+} + OH^{-}$$

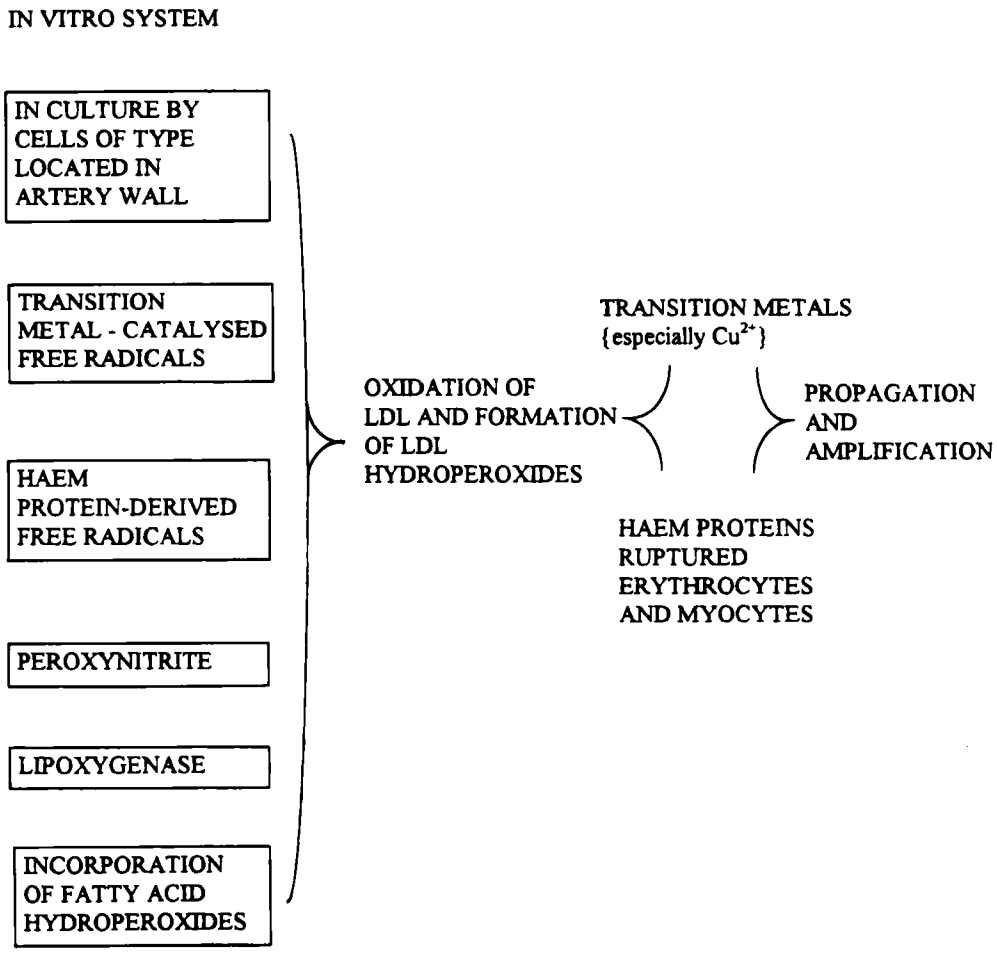

FIGURE 2 *In Vitro* Approaches to the Induction of LDL Oxidation

This process does not occur instantaneously in most cases because of a "resistance" to oxidation presumed to be due, at least in part, to the presence of antioxidants, particularly that of α-tocopherol. It has been calculated, based on extensive studies, that α-tocopherol contributes 30% to the lag phase to oxidation of LDL.[23] The first event in this process appears to be the progressive disappearance of the constituent antioxidants in an order which will be dependent on their reactivity, taking into account their ability to recycle or be regenerated by other antioxidants. Since β-carotene has an intense yellow colour, its consumption is marked by the loss of colour.

As this so-called "lag" phase is completed, the propagation phase begins with the acceleration of the oxidation of the polyunsaturated fatty acids, primarily linoleic acid and arachidonic acid, with amplification of conjugated diene (or triene) formation. This is a convenient marker for following the process (see below). Soon afterwards there is the appearance of the major oxidation products of fatty acid oxidation, the lipid peroxides and also a variety of aldehydes and hydroxyaldehydes, some of which are also readily measurable (see below). The appearance of most of these products reaches a peak after a few hours and they degrade or react with the protein contituents of the LDL.

Other lipid products are also formed, particularly lysophosphatidylcholine which originates from a phospholipase A_2 type activity on the principal phospholipid class, phosphatidylcholine, after it has become oxidised. Although this is associated with the oxidation process, LPC is also present in small amounts in native LDL, but there is a progressive accumulation of this lipid as oxidation proceeds. Other lipid oxidation products include a variety of sterol oxides derived from the oxidation of cholesterol, but again this is cumulative and they do not appear to degrade further.

The point is often made that Cu^{2+}- or Fe^{3+}-mediated oxidation is not physiological and that the ions bind to certain amino acid residues of apolipoprotein B-100, which may give rise to artefacts. One of the main advantages of the method is that the oxidising agent is a reasonably consistent "off the shelf" preparation which is simple to prepare. Furthermore, the lipid products that are generated are very comparable to those made by cell-induced oxidation in culture (see below) and these provide the basis for many of the tests relating to the extent of oxidation. This makes the procedure appropriate for application in the clinical setting. It is accepted that the effects of oxidation of the protein moiety should be treated with caution. The extent of oxidation of the lipids may be varied not only by time, but by choice of metal ion: ferric ions oxidise LDL more slowly. These variations may be particularly useful for biological experiments.

A number of factors need to be considered in order that the procedure may be standardised. These are as follows:

1. The concentration of the LDL
2. The purity of the LDL
3. The concentration of metal ions
4. The temperature at which the oxidation is performed
5. Cleanliness of the cuvettes
6. The nature of the buffer, for example, the use of iron with phosphate buffer is often questioned due to the potential for chelation; or the level of EDTA in the final concentration of LDL in relation to the copper concentration applied

The effects of these variables is outlined in the section on the measurement of conjugated dienes (see below). Other measurements such as thiobarbituric acid-reactive substances (TBARS), lipid peroxides, and also lysophosphatidylcholine may be made at fixed time intervals. Measurement of lipid peroxides and TBARS may be misleading because of the multiphasic nature of their appearance and disappearance. Other measures include the disappearance of lysine residues using trinitrobenzenesulphonic acid or changes in the uptake of the lipoproteins by macrophages (as described below). These measurements are of importance in experimental work, but do not lend themselves to continuous measurements, requiring more complex techniques or having greater inter-experimental variation.

As indicated above there is a common objection to the use of metal ions in such studies because they are perceived as unphysiological and may introduce artefacts. Furthermore, they lead to a profound oxidation of the lipoproteins. This can be reduced by lowering the concentration of cupric ions or replacing with ferric ions and also by terminating the oxidation by addition of large amounts of chelators, typically EDTA or DTPA in conjunction with a powerful antioxidant such as butylated hydroxytoluene (BHT). It should be noted, however, that copper and iron have been detected in "gruel" from atherosclerotic lesions,[24] although whether these are released as a consequence of the disease or available during its development is unclear as yet.

A gentler approach is to expose the lipoproteins to air at 37°C for 48 hours, or for longer periods if required, under sterile conditions. This is enough to initiate the earlier stages of oxidation so that the product is defined as minimally modified LDL with elevated concentra-

tions of lipid peroxides, but no change in the protein structure or electrophoretic mobility. This is a very useful research tool, but may have little practical value in terms of standard testing of individual LDL samples. The rates of oxidation may also depend, to some extent, on the presence of trace amounts of metal ions in the buffers or even water. It should also be stressed that oxidative modification of proteins may occur independently of lipid oxidation through direct effects of metal ions participating in mechanisms leading to amino acid oxidation, eg. lysine, tryptophan, cysteine.

2(ii) LDL Oxidation by Iron in the Absence of Cells

Iron ions by themselves are poor at oxidising LDL at pH 7.4. However, they are much more efficacious at lower pH, even at mildly acidic pH 6.5.[25] Iron is quite effective at oxidising LDL at pH 7.4 in the presence of thiols, e.g. cysteine, glutathione, mercaptoethanol or homocysteine.[26] In connection with this, it is of great interest that LDL oxidation by cells may depend on the uptake of the disulphide amino acid cystine into the cells, reducing it intracellularly to the thiol cysteine and then releasing this into the medium.[27] Cell-free LDL oxidation by a combination of iron and a thiol has been little used, however, to measure the oxidisability of LDL or to generate oxidised LDL for studies on its effects on cells.

In our hands, the *reproducibility* or intra-assay precision within experiments of the oxidation of LDL in a simple phosphate buffer by iron ions plus a thiol is less than that with copper alone when conjugated dienes are measured (Leake *et al.*, unpublished observations). LDL can be oxidised by iron/cysteine in Ham's F-10 medium in the absence of cells, with the oxidation being measured by macrophage uptake[28] or relative electrophoretic mobility,[29,30] however, with acceptable reproducibility within each experiment.

2(iii) LDL Oxidation by Haem Proteins

Haem proteins can oxidise LDL in two ways

(i) formation of initiating species through the activation with hydrogen peroxide by two oxidising equivalents to the ferryl myoglobin radical species.[2,31]

$$HXFe^{III} + H_2O_2 \rightarrow {}^{\bullet}X[Fe^{IV} = 0] + H^+ + H_2O$$

in which one oxidising equivalent is on the iron forming the iron(IV)-oxo ferryl haem radical and one oxidising equivalent is on the globin protein portion of the molecule. Haem proteins in the iron(II) state may also be activated by two oxidising equivalents, forming the iron(IV)-oxo ferryl haem protein radical. Such initiating species may readily oxidise polyunsaturated fatty acids in biological systems, whether by the ferryl myoglobin tyrosyl radical[32] (ie. Compound I form) or the ferryl myoglobin species (Compound II form).[33]

Haem proteins need not be activated to the radical form to mediate LDL oxidation. Haem proteins are capable of catalysing the peroxide-dependent oxidative and reductive decomposition of minimal amounts of pre-existing lipid hydroperoxides within the LDL particle[34] through their redox properties:

$$LOOH + HXFe^{III} \rightarrow LOO^{\bullet} + HXFe^{II}$$
$$LOOH + HXFe^{II} \rightarrow LO^{\bullet} + HXFe^{III}$$
$$LOOH + HXFe^{III} \rightarrow LO^{\bullet} + HX[Fe^{IV} = 0]$$

the latter mechanism being the most likely.[35]

The advantages of this mode of oxidation by haem proteins in the iron (III) oxidation state, such as metmyoglobin and methaemoglobin, are that decomposition of peroxide is the only mechanism of oxidation and no direct interactions with the protein take place. For example, copper is capable of involvement in mechanisms inducing direct protein oxidation. Furthermore, metmyoglobin-dependent oxidation is not influenced by concerns of EDTA concentration nor copper chelation affecting the true pro-oxidant concentration. The underlying tenet of the peroxide-dependence of

metmyoglobin-mediated LDL oxidation and lack of interference of protein oxidation etc. makes this haem-protein dependent system useful for studying the effects of novel drugs or dietary constituents in enhancing the resistance of LDL to oxidation. This is of particular significance where phenolic components capable of chelating copper or iron ions are concerned, such as those incorporating catechol-type or gallate structures, including phenylpropanoids,[36,37] flavonoids[38,39] and hydroxybenzoates.[40] When applying metmyoglobin as the pro-oxidant, the difference spectrum between metmyoglobin and LDL and that of the oxidising LDL is monitored. The conventional lag phase as seen with copper-induced oxidation is substituted by a gradual slope indicating a very slow oxidation in this early phase; however time to 50% maximal oxidation correlates directly with the observed lag-phase.

3. LDL Oxidation by Cells

LDL can be oxidised by cells, as mentioned previously, and, in fact, this is how oxidised LDL came into prominence.[18,41] It should be noted that cells simply speed up the oxidation of LDL that would have happened slowly anyway in the culture medium alone.[41,42] The rate of oxidation of LDL by macrophages depends on whether or not they are activated. Thus mouse peritoneal macrophages activated by γ-interferon plus lipopolysaccharide oxidise LDL far more slowly than nonactivated macrophages do, because the nitric oxide generated by activated macrophages inhibits LDL oxidation.[43]

The choice of the tissue culture medium for oxidising LDL is critical. Most workers use Ham's F-10 medium to incubate their cells with when they are oxidising LDL.[14–16,18,43–45] Ham's F-10 medium contains 3 μM $FeSO_4$ (at least it is added by the manufacturers in this form), 10nM $CuSO_4$ and 100 nM $ZnSO_4$. The presence of iron in this medium is essential for the oxidation of LDL to take place.[15] One of the authors (D.S.L. and colleagues) routinely adds extra iron (3 μM $FeSO_4$) to the Ham's F-10 medium to give a final concentration of 6 μM. This extra iron sometimes (but not always) increases the oxidation of LDL by cells, but does not appear to increase the oxidation of LDL in the cell-free wells very much. The addition of extra iron is therefore optional. The concentration of copper in Ham's F-10 medium (10 nM) is too low to catalyse LDL oxidation by cells although higher concentrations (0.5–1 mM) can do so.[45] Zinc inhibits LDL oxidation by cells in Ham's F-10 medium with 50% inhibition occurring at about 1–5 mM, depending on the experimental conditions.[46] The normal concentration of zinc in Ham's F-10 medium (100 nM) would be expected to have little effect, however, on the oxidation of LDL.

An important factor in culture medium as regards LDL oxidation is the concentration of phenol red, the coloured pH indicator. Its concentration in Ham's F-10 medium can be 12 mg/l or 1.2 mg/l. In fact, during the course of one study,[46] it was reduced by the manufacturers from 12 to 1.2 mg/l. Extra phenol red solution is therefore added to the Ham's F-10 medium to restore its level to 12 mg/l, as the concentration of phenol red can sometimes (but not always) affect the degree of LDL oxidation:[47] too much will inhibit LDL oxidation by cells, too little will increase oxidation in cell-free wells by iron. Thus caution must be exercised under these conditions. There is also some unknown difference between the Ham's F-10 medium supplied by different manufacturers, with medium supplied by some manufacturers supporting LDL oxidation much better than that supplied by others.

Some workers oxidise their LDL by cells in RPMI 1640 medium.[48,49] This contains no added transition metal ions, but it is possible that some are present as contaminants in the water used to prepare the medium or more likely in the salts used to make it. It is also possible that the cells themselves may release transition metal ions.[49] Other workers have reported that cells fail to oxi-

dise LDL in RPMI 1640,[41] consistent with the authors' observations. Dulbecco's modified Eagle's medium contains 0.25 μM $Fe(NO_3)_3$ but will not support LDL oxidation by cells.[15,41] LDL can be oxidised by cells in Hanks' balanced salt solution containing added 6 μM $FeSO_4$ but only when the pH is lowered to 6.25 or below.[25]

Serum is a very good inhibitor of LDL oxidation, with 1% (v/v) fetal calf serum inhibiting LDL oxidation by cells by about 50%.[15] Cells cultured in medium containing serum should therefore be washed in serum-free medium before they are incubated in serum-free medium containing LDL, otherwise traces of serum may inhibit the oxidation.

The concentration of LDL that is added to the cells is critical. The lower the concentration of LDL, the more oxidation will be obtained. If very low concentrations of LDL are used, for instance 20 μg LDL protein/ml, substantial oxidation may well occur in the Ham's F-10 medium in the cell-free wells and there may be no apparent difference in the oxidation of the LDL in the wells containing cells and in those without.[41] Many laboratories oxidise their LDL at 100 μg protein/ml, since at these concentrations the oxidation by cells is much faster than in the cell-free wells[37] and the kinetics of oxidation follow a well-defined and consistent pathway, as regards the formation of lipid hydroperoxides, thiobarbituric acid-reactive substances, oxysterols, electrophoretic mobility and the generation of a form of LDL that is taken up rapidly by macrophages.[50,51] The average concentration of LDL in plasma in the western world is about 700 μg protein/ml and the concentration of LDL in the interstitial fluid of the human normal aortic intima may be about twice this.[52] The way in which cells oxidise LDL at these high concentrations is therefore an important question, but little is known about the mechanism. It may be of interest, however, that the oxidation of a high concentration of LDL (5 mg protein/ml) by copper ions alone is slow and the kinetics are very different from those at 100 μg protein/ml.

The oxidation of radiolabelled LDL is faster than that of nonlabelled LDL because the radioactivity increases the levels of lipid hydroperoxides in the LDL during its radioiodination, storage or oxidation.[53] The extent of oxidation of LDL by cells will therefore depend on whether or not the LDL is radiolabelled and for how long the radiolabelled LDL has been stored before it is added to the cells.

In experiments where LDL is oxidised by cells, it is also essential to incubate LDL in cell-free wells as a control, as the oxidation of LDL occurs slowly in Ham's F-10 medium alone.[44] The LDL oxidised by cells is usually termed "cell-oxidised LDL" or "cell-modified LDL" and that in the cell-free wells is termed "control LDL". It is advisable to incubate the cell-oxidised and control LDLs in the same volume and in the same type of well, as these factors may conceivably affect the rate of oxidation observed. It is also important to include "native LDL" controls in these experiments, i.e. LDL that has not been incubated with cells, but has simply been stored at a high concentration at 0–4°C. The extent of oxidation of the cell-oxidised LDL, control LDL and native LDL can then be compared. This can sometimes be important because, if there is no difference in the oxidation of the cell-oxidised LDL and the control LDL, the investigators may be unsure whether the cells have failed to oxidise the LDL or whether any observed oxidation was due to spontaneous oxidation not mediated by cells (as can happen under certain conditions[41]). Comparing the control LDL to the native LDL would allow these two possibilities to be distinguished. The time of incubation of LDL with cells to induce fairly large scale oxidation is usually 18–24 h.

4. LDL Oxidation by Thermolabile Azo Compounds as Peroxyl Radical Generators

LDL oxidation can be studied independently of transition metal ions using azo initiators at 37°C. 2,2'-Azobis(2-amidinopropane)dihydrochloride (AAPH or ABAP) is water-soluble and will gen-

erate peroxyl radicals within the aqueous phase, whereas 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), is lipid-soluble and may generate peroxyl radicals within the LDL particles.[54,55] Azo initiators generate peroxyl radicals directly by temperature-dependent unimolecular decomposition. Although they have no relevance in vivo, they are good models for studying mechanisms.

The reaction sequence:

$$A-N=N-A \rightarrow A^{\bullet} + N_2 + A^{\bullet}$$
$$A^{\bullet} + O_2 \rightarrow AOO^{\bullet}$$
$$AOO^{\bullet} + LH \rightarrow AOOH + L^{\bullet}$$
$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$
$$LOO^{\bullet} + LH \rightarrow LOOH + L$$

where A = HCl.HN = C (NH_2) C $(CH_3)_2$ for AAPH and A = $(CH_3)_2$ – CH CH_2 C (CH_3) CN for AMVN. The advantage is that the rate of generation of radicals is virtually constant for the first few hours.

AMVN is added in ethanol, giving a final concentration of ethanol of about 1% (v/v). Some studies have not included EDTA in the incubation mixture,[55] but it can be added if desired to prevent the breakdown of lipid hydroperoxides by contaminating iron or copper in the phosphate buffer. The concentration of AAPH used may vary from 0.5 mM to 10 mM and of AMVN from 0.5 – 2 mM. The oxidation of the LDL may be followed by the conjugated dienes formed at 234 nm but, if so, it must be realised that AAPH has a relatively high absorbance below 260 nm; the A_{234} of a 2 mM solution is about 0.9 at the start of the incubation and about 2.8 after 18 h at 37°C. If LDL is used at 100 μg protein/ml, the increase in absorbance due to the formation of conjugated dienes will peak at about 0.6–0.7 at about 10–15 h with 2 mM AAPH, giving a final absorbance in excess of 3. This corresponds to a light transmission of less than 0.1% and it is asking a lot of a spectrophometer to measure this accurately. Higher concentrations of AAPH than 2 mM cannot therefore be reliably used if the A_{234} is being monitored. It is important to note that the absorbance of AAPH in the UV region changes as it thermally decomposes due to the change in structure from the azo chemistry to peroxide. Hence, it must be present in a reference cuvette in the absence of LDL and its absorbance subtracted from that of the test cuvette containing LDL and AAPH at each time point.

When applying AMVN, the lipophilic azo initiator, it is always assumed that it incorporates solely into the inner core of the LDL, generating peroxyl radicals in that location. This has not been demonstrated to be the case unequivocally. It is interesting to note that the interaction of AMVN with LDL has little influence on apoB modification.[55] It is unclear whether this implies that the protection of the cholesteryl esters from oxidation is completely independent of the protection of the phospholipids, their breakdown products and the latter's influence on modifications to the charge on the apoprotein B-100.

5. Peroxynitrite

Nitric oxide is released from a number of different cell types in response to different stimuli, including cells associated with the arterial wall and atherosclerosis, namely endothelial cells and macrophages. These cells also release superoxide anions, which are usually destroyed by superoxide dismutase. However NO and superoxide anions readily form a powerful oxidant, peroxynitrite. Peroxynitrite reacts with both sulphydryl groups in proteins and also tyrosine residues to form nitrosylated and nitrated products. Both nitrosothiols[56] and nitrated proteins[57] are found in the circulation even of healthy individuals. However, peroxynitrite will also oxidise lipids and there have been direct demonstrations that it will oxidise LDL.[58] There is no clear evidence that this has any pathophysiological relevance, but since macrophages form peroxynitrite, there is strong reason for considering it. It is moderately difficult to use in a routine context, since once prepared it needs to be kept at high pH (it rapidly breaks down at physiological pH) and care needs to be taken to check the pH of the LDL once the

reagent is added. For this reason it is likely to remain a method primarily of interest in basic research. Furthermore, contamination with NO_2^-, H_2O_2 and metals can influence and disturb the results, thus controls consisting of decomposed peroxynitrite are also essential here.

6. Glucose

Even in healthy individuals some of the lysine residues in LDL are glycated by the formation of a Schiff's base (approx 0.5 mol glucose/mol LDL) which may be increased up to 2 mol/mol in diabetes.[59] These may form advanced glycation products which may contribute to the atherogenicity of these particles. However, glucose in solution may also form reactive oxygen species (hydrogen peroxide and hydroxyl radicals), initially by the formation of an enediol from the open form of glucose. This autoxidative process is dependent on the presence of trace amounts of catalytic transition metals. Even glucose bound to proteins as the Schiff's base may generate ROS via enediols without the formation of the open-chain form of glucose.

These reactive species cause direct oxidation of lipoproteins, although the rate at which this occurs will depend on the addition of low concentrations of metal ions. "Slow" oxidation in the presence of 5–500mM glucose will occur to yield lipid peroxides, oxidised sterols and changes in tryptophan fluorescence over a period of days. The addition of 0.5μM Cu^{2+} will accelerate these actions of glucose and changes in electrophoretic mobility of LDL. These procedures are still experimental and relatively slow for routine assays.

IV COMPARISON OF METHODS OF MONITORING OXIDATION

1. Conjugated Dienes

The conjugated diene assay (usually at 234 nm) is a very convenient assay for the continuous monitoring of the oxidation processes by uv spectophotometry.[60] The method is reasonably accessible to most laboratories without extraordinary investment in special equipment. Most high grade (usually dual beam) spectrophotometers are suitable, but the process is made much easier if they are equipped with an automatic cell changer which are sold commercially with up to 10 cuvette positions. They must also have good software for data handling so that the large amount of data may be downloaded conveniently. The measurement may be translated into a number of parameters, the most important of which is the lag time measurement (when Cu^{2+} is used as pro-oxidant) devised by Esterbauer. An alternative is the time required to produce 50% of maximum oxidation which may be helpful if samples are very susceptible to oxidation. The lag time can be estimated with appropriate software programmes, which determine the point of intersection between the tangents to the lag phase and the propagation phase, the characteristic of a given sample of LDL to a specific challenge oxidation by Cu^{2+} under precise conditions. The assay itself is relatively simple and gives reproducible data (Fig. 3). In all cases, the samples should be run in duplicate and compared to a control without Cu^{2+} to monitor for any spontaneous oxidation.

It should be noted, that whereas most LDL samples isolated from normal plasma develop a maximum in the region of absorption of conjugated dienes of 234 nm, in some instances the peak is slightly red-shifted. This does not influence the time course plot for change in absorbance at 234 nm as compared with that at 238 nm. However, more severe changes have been observed in samples from patients with clinically diagnosed atherosclerosis, where direct measurement of A_{234} as a function of time of oxidation would have given an inappropriate indication of lag phase duration.[61,62]

It should be stressed again that the concentration of the LDL strongly influences the rate of oxidation even if the ratio of LDL to metal ions is

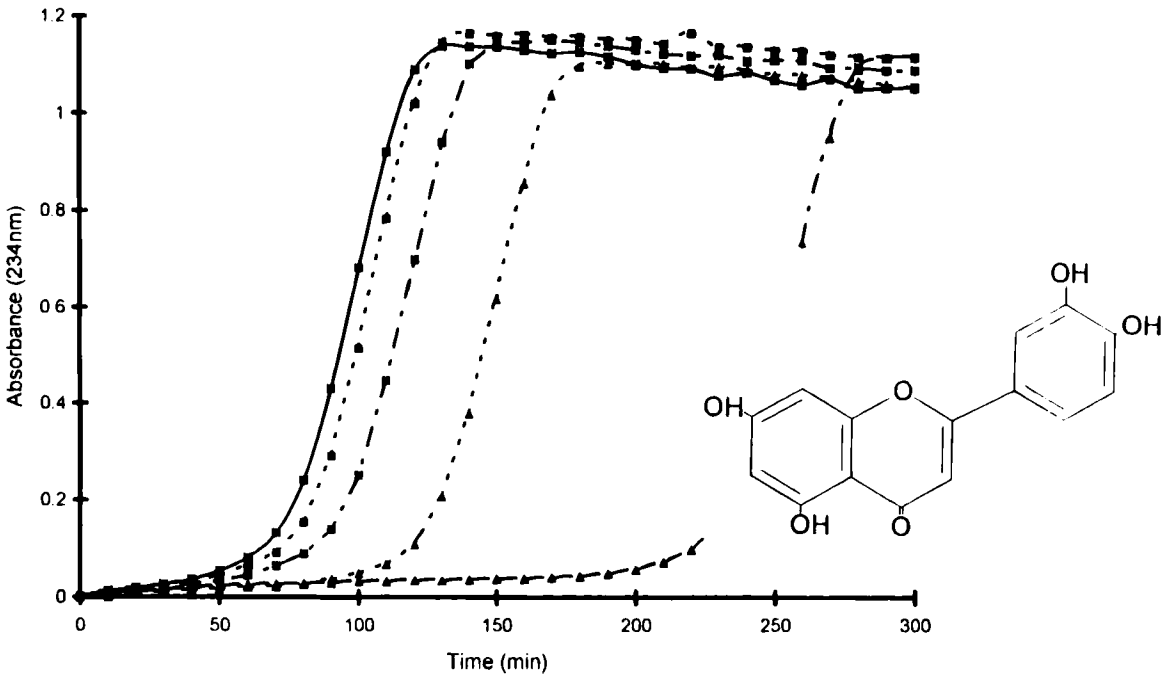

FIGURE 3 Time course of conjugated diene formation on oxidation of LDL (62.5 μg LDL protein/ml) mediated by Cu^{++} (1.6μM) and the dose-dependent influence of increasing concentrations of luteolin in prolonging the time to propagation of oxidation (30°C). Concentration of luteolin: 0.1, 0.25, 0.5, 1μM. [Brown and Rice-Evans, unpublished observations].

kept constant. As the concentration increases, the rate at which the oxidation occurs per mg of LDL decreases. The concentration recommended by Esterbauer is of the order of 0.1 μM, 50 μg LDL protein per ml: it should be emphasised that the total weight of the LDL is five times that of the protein component alone. To estimate this an assay of cholesterol may be performed and an assumption made that this represents 31.6% of the total weight of LDL. This concentration of LDL can be extended to 100 or 125 μg/ml, in the authors' views, in the case of copper as pro-oxidant (Fig. 3). The cupric ion concentration should then be 1.66μM. Since the process is temperature-dependent, the recommended temperature is 30°C in a regulated cuvette holder so that in cases where the lag time is short, more accurate determinations can be made (compared to 37°C). Peltier electronics give more efficient temperature stabilisation and more highly reproducible results than a circulating waterbath system. The measurements may be made against air and a separate control of LDL without Cu^{2+} run in parallel. Alternatively a difference spectrum may be measured, subtracting initially the spectrum of the native LDL solution. The cuvettes should have a path length of 1 cm and accuracy is increased if they are capped to prevent evaporation. Applying haem proteins such as metmyoglobin to induce LDL oxidation, the initial spectrum of metmyoglobin must also be subtracted in addition to that of the LDL. For example, with 125 μg/ml LDL, 5 μM MetMb is usually applied in place of 1.66 μM Cu^{++}. It should be noted that as haem proteins and azo initiators contribute to the absorbance, it is preferable to use a 2mm cuvette with these latter pro-oxidants. Cleaning of the cuvettes in reagents rich in metal ions e.g. chromic acid should be avoided. Buffers containing high concentrations of phosphate should not be used since iron phosphates may precipitate out and

confound the measurements. The data may be captured and processed with programmes such as Excel.

Efforts are now being made to simulate the process of lipid peroxidation in low-density lipoproteins by setting up a minimal kinetic model based on equations which represent a number of the reactions known to occur in this process.[63] The model suggests that there is significant antioxidant activity which cannot be attributed to the known endogenous antioxidants, primarily α-tocopherol.

2. Anion-Exchange Chromatography

LDL oxidation can be monitored by anion-exchange chromatography (FPLC using Mono Q HR5/5) utilising multistep NaCl gradient elution.[64] This method detects 5 fractions of LDL, with the more negative forms being the more oxidised. This technique allows the extent of LDL oxidation to be assessed by comparing the relative sizes of the different peaks, but requires specialist equipment and takes almost one hour to analyse each sample, and so would not be suitable for high throughput work.

3. Electrophoresis

(i) Agarose Gels

LDL normally has a net negative charge, with an isoelectric point of 5.4.[65] Upon oxidation, LDL acquires a greater net negative charge, partly due to the neutralisation of the positive charges of the lysyl residues of apo B-100 as aldehydes bind to these. This provides an easy and reliable way to measure the extent of oxidation of LDL, particularly if an indication of the modification of apo B-100 is required. It is noteworthy that numerous groups have found that the entire LDL band migrates towards the anode faster when it is oxidised, rather than one band being seen for native LDL and one for oxidised LDL,[19] probably due to the formation of aggregates.

The type of gel that is used can make a surprising difference to the results that are obtained, in that it can affect not only the absolute rate of migration of LDL or oxidised LDL but also the percentage increase in the migration of oxidised LDL compared to native LDL that is observed. A particularly useful gel is the Paragon Lipo gel from Beckman. These gels consist of 0.5% agarose and the samples (3–5 μl) are applied to the gels using a template and are allowed to diffuse into the gel for 5 min or more before running at 100V for 30 min in diethylbarbituric acid buffer, pH 8.6. The LDL is then fixed in the gels for 5 min using a mixture of ethanol, water and acetic acid. The gels are dried and stained for 5–10 min with a lipid stain, Sudan Black B in 55% (v/v) ethanol, destained in 45% (v/v) ethanol for a few minutes, rinsed in water and dried. The relative electrophoretic mobility of the oxidised LDL can then be calculated, i.e the distance migrated by the oxidised LDL divided by the distance migrated by the native LDL. Samples of LDL at a concentration of the order of 100 μg protein/ml can be applied to the gels (Fig. 4)[66] although some other systems require far more concentrated LDL for it to be readily visualised.

There is usually a lag period of several hours with macrophages or Cu^{2+} before the electrophoretic mobility is increased.[51] The mobility then increases and remains stable, in contrast to measurements of lipid hydroperoxides which increase after a lag period, peak and then decrease. The relative electrophoretic mobility is therefore a valuable measure of the later stages of LDL oxidation.

(ii) Polyacrylamide Gel Electrophoresis

Apo B-100 in LDL is fragmented during its oxidation into a large number of polypeptides due to oxidative scission.[67] LDL oxidation can therefore be followed by the disappearance of the apo B-100 band of M_r 513000 and the appearance of bands of lower M_r by sodium dodecyl sulphate-

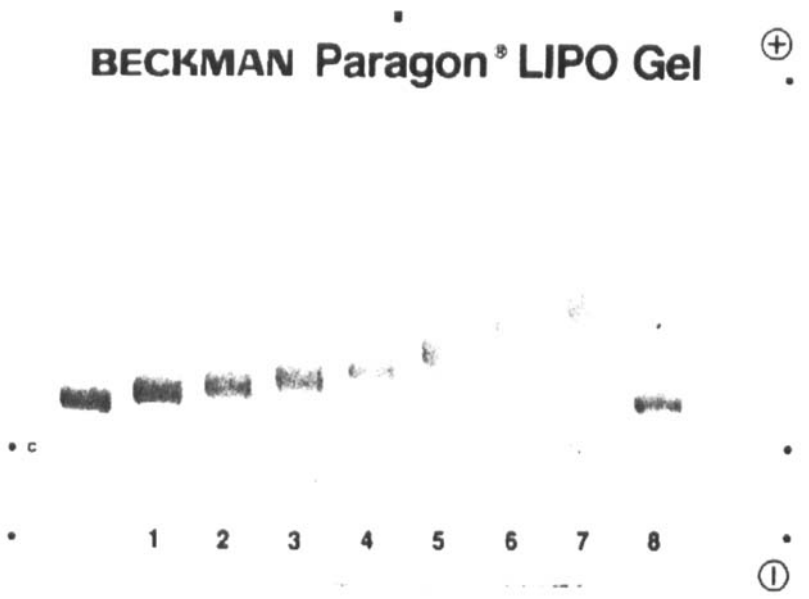

FIGURE 4 Agarose gel electrophoresis of the time-course to the oxidation of LDL (0.5 mg/ml) mediated by oxymyoglobin: LHS, native LDL; Lanes 1–7 after incubation for 1–7h respectively.[66]

polyacrylamide gel electrophoresis. However, this is a time consuming way to measure LDL oxidation and only a limited number of samples can be processed. Some workers have noticed that the Mr of apo B-100 can increase, as well as decrease, during oxidation, due to the covalent cross-linking of apo B-100 molecules in two or more LDL particles.[10]

4. Monitoring LDL Oxidation by Fluorescence

The fluorescence of LDL increases when it is oxidised and the fluorescence spectra are similar for LDL oxidised by copper, iron or cells.[68] The fluorescence can be measured at an excitation wavelength of 360 nm and an emission wavelength of 430 nm and at these wavelengths is probably due to the reaction of 4-hydroxynonenal or similar aldehydes with apo B-100 to generate an unknown fluorophore.[68,69] An advantage of this technique is that the increase in fluorescence of the LDL upon oxidation can be measured continuously, as is the case with conjugated dienes. The fluorescence increases, after a lag period, at about the same time as the conjugated dienes increase but it continues to increase after the conjugated dienes have peaked, presumably because the conjugated diene lipid hydroperoxides fragment to form aldehydes which then react with the apo B-100 to form fluorophores.[5] Unlike the conjugated dienes, which peak and then decrease, the fluorescence will eventually plateau; this would be an advantage over measuring the conjugated dienes if only a single time point is taken.

5. Lipid Hydroperoxides

The xylenol orange assay[70,71] or Fox assay describes a sensitive spectrophotometric system for detecting authentic peroxides and has been successfully applied to the measurement of lipid hydroperoxides in low density lipoprotein. Hydroperoxides oxidise ferrous to ferric ion in dilute acid and the resultant ferric ions are determined using ferric sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange [o-cresolsulfonephthalein 3'3"-bis

(methylimino) diacetic acid sodium salt] binds ferric ion with high selectivity to produce a coloured (blue-purple) complex with an extinction coefficient of 1.5×10^4 M^{-1} cm^{-1} and absorbance maximum of 560nm. The method compares favourably with the iodometric assay, TBA assay and conjugated diene measurement. No extraction step is required for analysis of lipoprotein in the 90% methanol/25mM H_2SO_4 environment in which the assay is performed.

Triphenylphosphine (TPP) is used as a specific reductant of hydroperoxides, converting them to the corresponding alcohol. This allows the measurement of authentic hydroperoxides reacting in the assay and removes any background signal generated. Each sample is therefore measured with and without TPP, the difference between the two being the lipid hydroperoxides in the sample. Background values for plasma have been reported to be high. The mean value obtained for native LDL in CR-E's laboratory = 13.3 ± 8.8 nmoles/mg LDL protein after analysis of LDL samples isolated from 47 donors on separate occasions, each assay performed in triplicate (Bourne, Castelluccio, Rodriguez, Sampson and Yang, unpublished observation). Lipid hydroperoxides can also be measured easily in LDL using the tri-iodide assay.[72] The lipids of LDL are dispersed by the detergent used in an enzymatic cholesterol assay kit and the hydroperoxides oxidise I^- to I_2 which is detected spectrophotometrically. The value given for native LDL for the iodometric method corresponds to 25 nmol/mg LDL protein in the original publication[72] and a mean value of 18.6 ± 9.4 nmol/mg LDL protein [range 10–30] in a subsequent publication by Esterbauer *et al.*[73] This sort of range was deemed to be at the borderline of the detection limit for the iodometric assay.[73] Others report levels of endogenous peroxide in freshly isolated LDL in the range of 10–20 nmoles/mg LDL protein[35] applying this method; and 22.3 ± 3.8 nmol/mg LDL protein [n = 6] in a prior publication.[74] The oxidation of LDL by AAPH is shown in Figure 5 as the comparison of increased formation of conjugated dienes with hydroperoxide formation (Holloway, Paganga and Rice-Evans, unpublished observations).

The hplc procedure for detecting lipid hydroperoxides has the advantage that the identity and mass of specific lipid peroxides may be determined down to very low concentrations, well below that available by the colorimetric methods. This sensitivity is dependent on the availability of chemiluminescence detection.[75] Stocker *et al*[76] have recently reported that LDL freshly isolated from healthy subjects was free from detectable amounts of cholesterol ester hydroperoxides and phospholipid hydroperoxides as measured by hplc with post-column chemiluminescence detection, suggesting that if lipid hydroperoxides are present at all, the levels must be below 1 nmol/mg LDL protein. This method of detection does require considerable dedication of technical resources and is unlikely ever to be suitable for any routine assays in large scale clinical research. In some cases, ultraviolet detection can be used but with lower sensitivity. A comment should be made on the wide-ranging differences between the detected endogenous peroxide levels in LDL applying the hplc/chemiluminescense method compared with the spectrophotometric assays. (A direct comparison of the methods has yet to be carried out in a single laboratory on the same LDL samples). No-one is sure whether the Fox and El-Sadaani methods are determining additional unidentified components or whether the hplc assay is missing some contributing features. On the other hand, it may relate to the methods applied for isolating the LDL; for example, applying the Fox assay in C R-E's laboratory after isolation by the rapid method gives 3 nmoles/mg LDL protein, compared to 13.3 as mentioned above using the sequential method.

It should also be mentioned that an equally sensitive method for the assay of oxidation has been developed by assaying the formation of isoprostanes. This seems to be even more equipment intensive, requiring detection by mass spectroscopy. However, it is a very sensitive indicator. Basal levels of F_2-isoprostanes in LDL

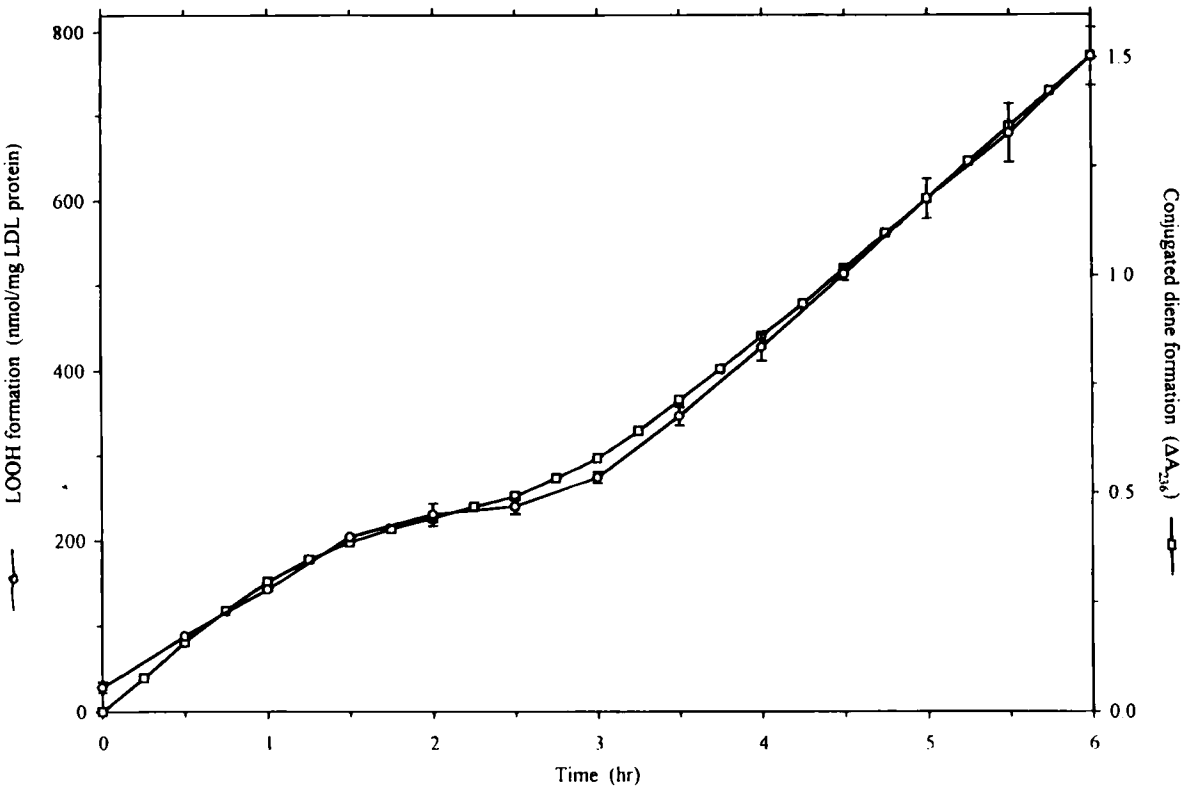

FIGURE 5 The time course of LDL oxidation mediated by the azo initiator AAPH. LDL (0.15mg/ml) was incubated at 37°C with 1.5mM AAPH in PBS, pH 7.4 (containing 10μM EDTA). Conjugated dienes were monitored in a 2mm cuvette. Lipid hydroperoxides were measured using the FOX assay; datapoints represent means ± SD. These conditions were set for a situation in which peroxides are produced at a constant rate and all the radicals from the azo initiator react with the LDL. This is indeed substantiated from the observed matching of the rates of conjugated diene formation with that of the peroxides.

are reported to be 2.6 ± 1.0 ng/mg LDL on 5 individual donors.[77]

6. Uptake of Oxidised LDL by Macrophages

The extent of oxidation of LDL can be assessed by measuring its rate of uptake by macrophages, which is in fact a type of bioassay. This assay was, in fact, the principal method by which cell-modified (oxidised) LDL was discovered[18] and the rapid uptake of oxidised LDL may be the process for the modification of macrophages into cholesterol-laden foam cells in atherosclerotic lesions. Despite its complexity, this method of measuring the extent of LDL oxidation is surprisingly reliable and shows low variability within experiments.

The uptake of ^{125}I-labelled LDL by macrophages is usually measured in terms of the radioactive degradation products appearing in the medium.[18] Most of the radioiodine in ^{125}I-labelled LDL is present on the tyrosyl residues of apo B-100. The ^{125}I-labelled LDL appears to be recognised by a variety of types of scavenger receptors on macrophages and is rapidly endocytosed and delivered to lysosomes. The apo B-100 is degraded by cathepsins B and D and the ^{125}I-monoiodotyrosine produced passes through carriers in the lysosomal and plasma membranes back into the culture medium, after a short lag phase due to the time taken for internalisation and proteolysis. After a number of hours or an overnight incubation, the amount of

^{125}I-iodotyrosine in the culture medium is a good measure of the amount of ^{125}I-labelled LDL that has been degraded by the macrophages.

^{125}I-labelled oxidised and control ^{125}I-labelled LDL is incubated with wells of macrophages and also in cell-free wells and the radioactive degradation products in the medium are then measured. The degradation products in the cell-free wells are subtracted from those in the medium of the macrophages. For control ^{125}I-labelled LDL, the radioactive degradation products in the cell-free wells will be very small, but for the oxidised ^{125}I-labelled LDL they will be greater, especially for cell-oxidised LDL. This is because the cells used to oxidise the ^{125}I-labelled LDL will have taken up some of the LDL, degraded it and released radioactive degradation products into the medium.

The medium in which the oxidised ^{125}I-labelled LDL is incubated with the macrophages is important, as there is an obvious need to prevent the macrophages from further oxidising the LDL. Dilution in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and an antibiotic (say 50 μg gentamicin/ml) is recommended, as macrophages cannot oxidise LDL in Dulbecco's modified Eagle's medium and 5% (v/v) foetal calf serum completely inhibits LDL oxidation of macrophages (in terms of increased macrophage uptake).[15] Although the presence of an antibiotic is essential under the conditions of these incubations, some antibiotics apparently show antioxidant properties; gentamicin is selected partly because it shows only a slight effect.

The concentration of the oxidised ^{125}I-labelled LDL can also be important, especially if the LDL has been oxidised by cells. The uptake of oxidised LDL by macrophages starts to plateau at concentrations greater than about 10μg protein/ml[15,19] and so the percentage difference between the degradation products produced by the macrophages and those already present in the oxidised ^{125}I-labelled LDL (due to uptake and degradation by the cells used to oxidise the LDL) will be greater if fairly low concentrations are used, thus improving the precision of the assay. Also, the oxidised LDL is less likely to be toxic to the macrophages if its concentration is fairly low.[78] The time of incubation of the LDL with the macrophages may also be important, especially with macrophage-oxidised LDL, as there may be substantial degradation products present due to the uptake and degradation of the oxidised LDL by the macrophages that were used to oxidised the LDL. If only low amounts of degradation products are present, a fairly short incubation time, say 5 h, may be all that is required, otherwise a 18–24 h incubation time may be required to increase the degradation products from the macrophages to a sufficiently high level compared to background.

A number of different types of macrophages can be used to measure the uptake of oxidised LDL. Resident mouse peritoneal macrophages are often used.[14,15] The peritoneal cells, obtained by the technique of Cohn and Benson,[79] are collected with a Pasteur pipette, centrifuged at 250 h for 10 min at 4°C and resuspended in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and gentamicin (50 μg/ml). A sample of the cell suspension is diluted in buffer and counted in a haemocytometer (not counting the red blood cells, which are smaller than the leucocytes). The rest of the cell suspension is then plated into tissue culture wells at the required number of cells per well. Note that only about half the peritoneal cells counted will be macrophages, the rest being mainly lymphocytes.[79] Only the macrophages will adhere and the other cells can be washed away from 1–2 h or after an overnight incubation. The macrophages can be used the same day or the following day.

A line of macrophage-like cells, J774 cells, derived from a mouse tumour can also be used.[18,80] These cells will grow in either suspension culture or attached to culture vessels. They can be grown in Dulbecco's modified Eagle's medium contain-

ing 20% (v/v) heat-inactivated foetal calf serum, L-glutamine (2 mM), penicillin (10 iu/ml), streptomycin (10 μg/ml) and amphotericin B (1 μg/ml). In our experience, their growth is quite sensitive to the culture medium used. They can be frozen in liquid nitrogen in 50% (v/v) fetal calf serum and 10% (v/v) dimethylsulphoxide for indefinite storage (Lamb and Leake, unpublished observations). The absolute rate of uptake of native and oxidised ^{125}I-labelled LDL is less than that by the mouse peritoneal macrophages, but the percentage difference between the two types of LDL is in general comparable for the two types of macrophage. The advantage of J774 macrophages to measure the rate of uptake of oxidised LDL is that they avoid the use of animals and are cheaper.

Human monocyte-derived macrophages can also be used.[81] Mononuclear cells (monocytes plus lymphocytes) are isolated from blood by centrifugation through Ficoll-Hypaque and plated in culture wells. The monocytes are allowed to adhere and the lymphocytes are then washed away. A disadvantage of the technique is that the cells need to be left for 10–14 days because the scavenger receptors on freshly isolated monocytes have only a low activity which increases rapidly between 5 to 9 days.[82]

The rate of degradation of oxidised ^{125}I-labelled LDL can be measured as the noniodide trichloroacetic acid-soluble radioactivity (^{125}I-monoiodotyrosine) in the medium by the method of Drevon *et al.*[83] This method relies on the intact ^{125}I-labelled LDL present being precipitated by trichloroacetic acid. Bovine serum albumin is added as a carrier to add bulk to the precipitate. When LDL is iodinated by the iodine monochloride method, small amounts of radioiodide ions apparently remain in the ^{125}I-labelled LDL preparation despite extensive dialysis and, although the amounts are small compared to the label incorporated covalently into the LDL particles, the amounts may be quite large compared to the ^{125}I-monoiodotyrosine released by the cells. The radioiodide ions can be easily removed, however, by adding $AgNO_3$, which leads to the formation of insoluble silver iodide.

$$Ag^+ + {}^{125}I \rightarrow Ag^{125}I$$

The chloride ions in the culture medium form insoluble silver chloride, which acts as a carrier for the tiny amount of silver iodide precipitate that is formed. It should, therefore, be checked that there is a molar excess of silver ions over the amount of chloride ions present. After the precipitation of the intact ^{125}I-labelled LDL and radioiodide ions, the ^{125}I-monoiodotyrosine in the supernatant can be measured in a gamma counter.

The original method of Drevon *et al.*[83] has been simplified and is reproduced here.[46] Each 16 or 22 mm well of macrophages or cell-free well contains 1 ml of medium and 900 μl of this is taken into a tube and its total radioactivity determined, as a final check that the amounts of radioactivity added to each well were the same. The tubes are then placed in ice and 100 μl of bovine serum albumin (30 mg/ml) are added (if the medium contains serum, the proteins in this will also act as carrier proteins).

Ice-cold 3M trichloroacetic acid (250 μl) is added and the tubes are vortexed. $AgNO_3$(250 μl of 700 mM) is then added and the tubes are vortexed again. The mixed precipitate of protein and AgI/AgCl is then sedimented (1500 g for 10 min at 4°C) and a sample (1 ml) of the supernatant is carefully removed and its radioactivity determined. The mass of ^{125}I-labelled LDL degraded per culture well can then be calculated, bearing in mind that the efficiency of a gamma counter depends on the counting geometry (0.1 ml of radioactive solution in the bottom of a tube will not give the same cpm as 0.1 ml of the same radioactive solution in the same tube if 0.9 ml of water is added to it and it is recounted) and thus a standard counting geometry has to be maintained or allowed for. Earlier methods for removing ^{125}I involved its oxidation to iodine by H_2O_2 and its extraction into chloroform, but this procedure is much more cumbersome than using $AgNO_3$ and is probably more hazardous.

After the removal of the medium from the target macrophages, the amount of cell protein is measured by washing the cells 3 times with Dulbecco's phosphate buffered saline containing Ca^{2+} and Mg^{2+}. (Three washes are required to effectively remove the serum proteins, as the amount of serum protein originally present in each well is far greater than the amount of cell protein present. If the washing procedure is carefully carried out, the amount of protein in the washed cell-free wells will be very small). The macrophages are dissolved in 0.7 ml of 0.2 M NaOH for 20 min at room temperature. This is enough time for all the cell protein to be solubilised.[84] (Surprisingly if NaOH solution at 37°C is used for longer than 30 min, less rather than more protein apparently is obtained[83]). The wells are then swirled to mix the dissolved proteins. This is important because the dissolved protein tends to form a viscous coating at the bottom of the well and requires encouragement to mix properly. The protein content of 0.5 ml of the lysate can then be determined by a Lowry protein assay or a suitable alternative.

Oxidised LDL is resistant to lysosomal proteolysis.[85,86] Therefore, if ^{125}I-labelled LDL is incubated with macrophages, about half of the radioactivity taken into the cells will accumulate[79,84] within their lysosomes. To estimate the true uptake of oxidised ^{125}I-labelled LDL by cells, the accumulated radioactivity within the cells should be added to the radioactive degradation products appearing in the medium.[87] The cell-associated radioactivity can be determined simply by counting the radioactivity in 0.1 ml of the NaOH lysate (with 0.5 ml out of the total of 0.7 ml being used for the protein assay, as described above), after adding water to bring the volume up to whatever is the standard counting geometry or alternatively using a simple mathematical correction to allow for the different counting efficiency. The small amounts of radioactivity in the "lysates" of the cell-free wells are subtracted from those in the macrophage lysates to allow for any ^{125}I-labelled LDL that remains bound to the plastic of the wells and is removed by the NaOH solution. A convenient way to display these results is as a histobar, whose height shows the total uptake of the ^{125}I-labelled LDL, with a horizontal line across the bar which shows the degradation products in the medium on one side of it and the cell-associated radioactivity on the other side.

There is a relationship between the electrophoretic mobility of LDL and its degradation by macrophages, but it is not a linear one. The generation of a form of LDL that is taken up faster by macrophages shows a longer lag phase than the increase in electrophoretic mobility does[44] (Fig. 6). After a lag phase, there is a rapid increase in the electrophoretic mobility of LDL for only a modest, if any, increase in macrophage uptake. Later on the electrophoretic mobility increases only slowly whilst there is a rapid increase in macrophage uptake. This indicates that the factors that cause the increased uptake of LDL by macrophages include more than just the net negative charge of the LDL particles. Measuring the uptake of LDL by macrophages will therefore measure something that electrophoretic mobility does not, in that it will detect the even later stages of LDL oxidation.

V THE GENERATION OF OXIDISED LDL WITH DEFINED EXTENTS OF OXIDATION

A method has been developed in the laboratory of DSL to oxidise LDL to various defined levels of oxidation. Freshly isolated LDL containing 100 μM EDTA is diluted to 100 μg protein/ml in sterile Dulbecco's phosphate buffered saline (137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4 and 8.1 mM Na_2HPO_4). (At higher concentrations of LDL, as mentioned above, the kinetics of oxidation are quite different and are poorly defined). $CuSO_4$ is added to give a net concentration of 5 μM above the final concentration of EDTA pre-

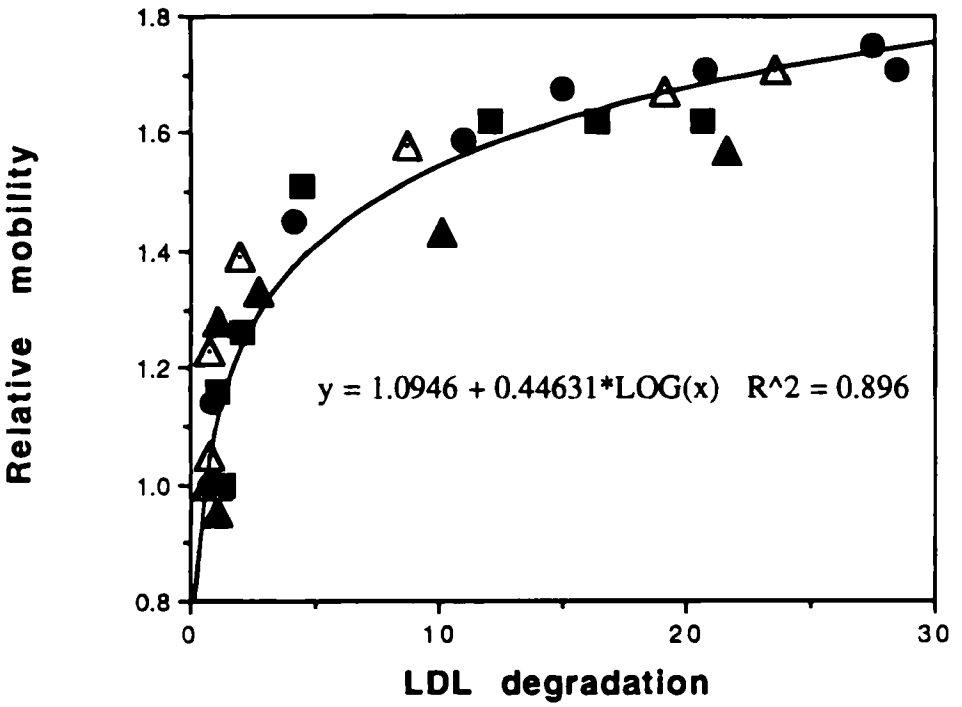

FIGURE 6 The relationship between the electrophoretic mobility and the rate of macrophage degradation of oxidised LDL. ^{125}I-Labelled LDL was oxidised by $CuSO_4$ (5 or 25 μM) or by mouse peritoneal macrophages (MAM) in a number of experiments. Its relative electrophoretic mobility in Ciba-Corning 1% (w/v) agarose gels (compared to native LDL) was then measured, as was its degradation by mouse peritoneal macrophages (which is expressed as μg LDL protein/mg cell protein in 18, 20 or 22 h). These two measures of LDL oxidation were then plotted against each other. Reproduced with permission. From ref 51.
△,▲ Cu^{2+}-oxidized LDL, 5 μM
● Cu^{2+}-oxidized LDL, 25μM
■ MAM-oxidized LDL

sent (usually about 1–2 μM). (In a simple phosphate buffer, EDTA binds and inactivates copper ions to inhibit LDL oxidation[88]). Care is taken to mix the LDL gently and not to vortex it, as this causes the aggregation of LDL.[89] The LDL is incubated at 37°C and its conjugated diene content is monitored every 10 min or so by transferring a sample to quartz cuvettes and measuring the absorbance at 234 nm. The sample is then transferred back to the rest of the oxidising LDL before the next sample is taken and in this way the whole of the LDL is oxidised to exactly the same extent. To form what we term "mildly oxidised" LDL, the A_{234} is allowed to increase by about 0.2 above the initial absorbance (about 0.5), which takes about 80–100 min. This corresponds to an increase in conjugated dienes of about 12% of the maximum level that will be obtained in the LDL. The oxidation is stopped with 1 mM EDTA and the mildly oxidised LDL is kept at 4°C. The oxidation of the rest of the LDL is allowed to continue up to its peak of A_{234} of about 1.5–1.9, which takes about 3 h. This is termed moderately oxidised LDL and will contain the maximum levels of lipid hydroperoxides. (Which closely follow the conjugated dienes during the early stages of LDL oxidation). The oxidation is again stopped by 1 mM EDTA and the LDL stored at 4°C. The rest of the LDL is left to oxidise for a total of 24 h, during which time the lipid hydroperoxide levels fall but later oxidation products are produced. EDTA is again added.

The oxidised LDLs need to be concentrated by ultracentrifugation before use in cell culture

studies, so that the culture medium is not diluted too much by the buffer the oxidised LDLs are prepared in. Solid KBr is added to increase the density to 1.2 g/ml. (The density of native LDL is 1.019–1.063 g/ml but this can increase considerably upon oxidation). The mass of KBr is calculated according to the following equation (the partial specific volume of KBr being 0.309 at a density of 1.2 g/ml).

$$\text{Mass of KBr} = \text{vol of sample (ml)} \times \frac{(1.2 - 1.006)}{1 - (0.309 \times 1.2)}$$

Chelex-100 is also added to chelate any contaminating transition metal ions present, especially in the KBr. The KBr takes about 3 h to dissolve by gentle stirring using a magnetic follower at room temperature. The LDL is then centrifuged at 250 g for 10 min to sediment the Chelex-100 and the supernatant is centrifuged at 40,000 rpm (149,000 g_{av}) for 18 h at 4 °C in 35 ml tubes. The LDL is removed from the top of the tubes using a needle and syringe and dialysed overnight at 4°C in the dark in dialysis tubing (previously boiled in EDTA solution) against 4 × one litre of 154 mM NaCl/16.7 mM NaH_2PO_4/21.1 mM Na_2HPO_4/100 μM or 1 mM EDTA, pH7.4. The LDLs are then sterilised using low protein binding filters of pore size 0.2 μm and assayed for protein using a Lowry or Markwell assay. Their relative electrophoretic mobilities are assessed using Beckman Paragon gels and their lipid hydroperoxides are measured using a triiodide method[72] or the Fox assay.[70,71] The lipid hydroperoxide levels in the native, mildly-oxidised, moderately-oxidised and highly-oxidised LDL are 0–70, 20–100, 500–800 and 0–300 nmol/mg protein, respectively, and the relative electrophoretic mobilities are 1.0, 1.1–1.2, 2.1–2.8 and 3.8–5.0, respectively. The oxidised LDLs should be used for the studies on cell function as soon as possible, but the lipid hydroperoxide levels are relatively stable at 4°C.

REASONS FOR INCOMPATIBLE COMPARISONS BETWEEN LABORATORIES

There have been a number of instances in which two laboratories have investigated the effects of oxidised LDL on a certain cellular activity and one laboratory has found a stimulation of that particular activity whereas the other laboratory has found an inhibition. One reason to explain this is that the extent of oxidation of the LDL may have varied between the laboratories. One oxidation product or products in LDL may have conceivably increased a certain cellular activity, whereas a different oxidation product or products may have decreased it (maybe by a cytotoxic effect) and the ratio of the various oxidation products may have varied between the two laboratories. The question arises as to which effect is the one most likely to occur in atherosclerosic lesions? (In fact, it is possible that both effects may occur, one in very early lesions which may contain only mildly oxidised LDL and the other in more advanced lesions where more highly oxidised LDL may occur).

It is therefore important that studies of the effects of oxidised LDL on cells should provide a reliable indication of the extent of oxidation of their LDL. Giving the exact details of the way in which the LDL was oxidised is, of course, also important, as the extent of oxidation may depend on:

(i) the nature of the oxidative stress and its concentration (copper ions, iron ions, cells, azo initiators, haem proteins and so on);
(ii) the concentration of the LDL (this has a profound affect on the oxidation obtained);
(iii) the exact composition of the buffer or tissue culture medium used (where relevant);
(iv) the time of incubation;
(v) the temperature of incubation and
(vi) whether the EDTA in the LDL preparations was removed or not (this can also have quite a large effect on oxidation either inhibitory or stimulatory[80]). Even if these factors are all standard-

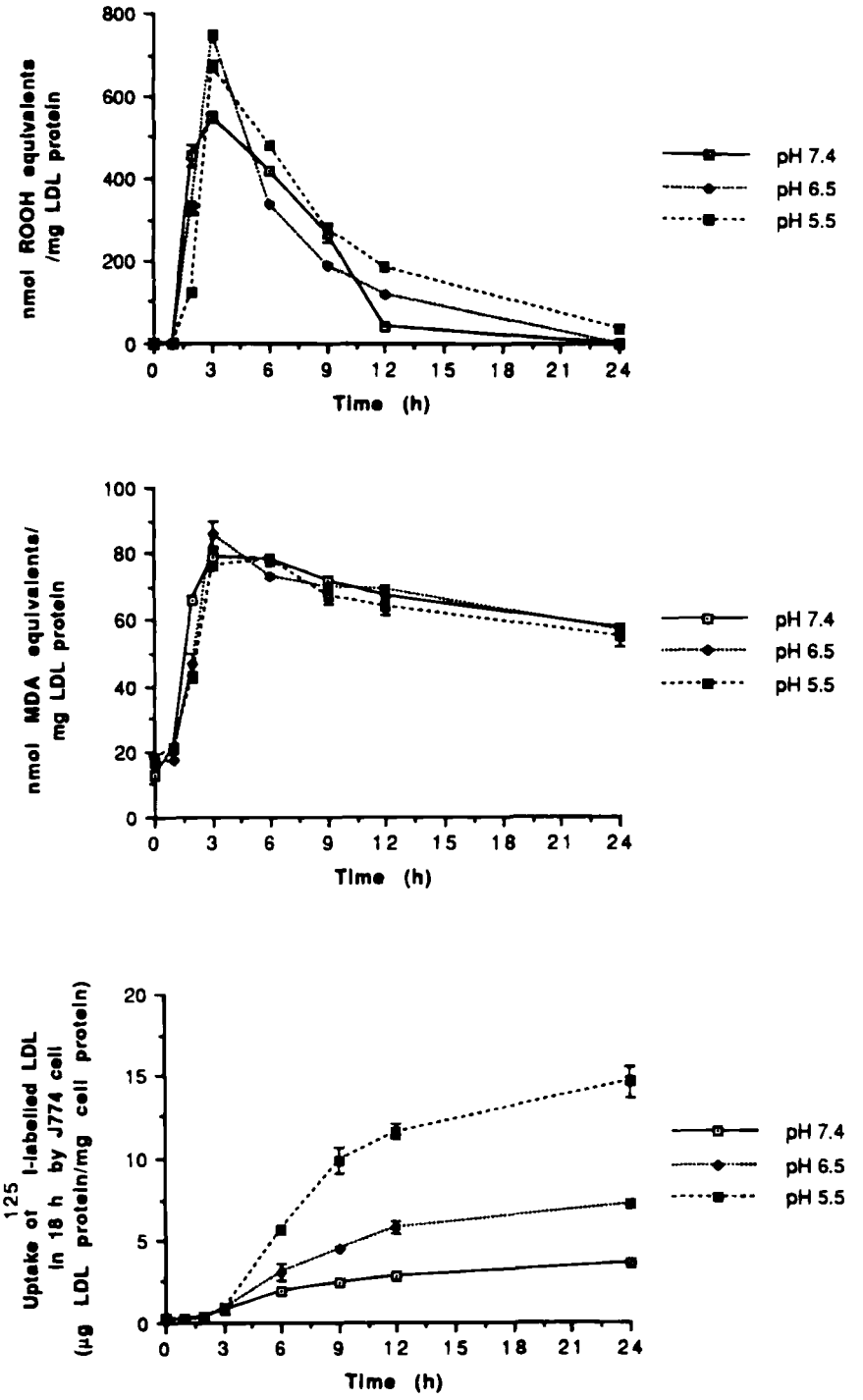

FIGURE 7 The time course of LDL oxidation by copper. ^{125}I-labelled LDL (100 µg protein/ml) was incubated at 37°C for upto 24 h with a net concentration of $CuSO_4$ of 5 µM (above the EDTA present) in a phosphate buffer of various pH values. Samples were collected into EDTA (100 µM) and butylated hydroxytoluene (20 µM) at various times to prevent further oxidation and assayed for lipid hydroperoxides, TBARS and the rate of uptake of the LDL by J774 macrophage-like cells. Each point represents the mean ± SEM of triplicate observations (sometimes the SEM cannot be seen if it is smaller than the symbols). Reproduced with permission from ref. 50.

ised, the extent of oxidation of LDL obtained in various laboratories may vary because of possible differences in the level of oxidation of the LDL at the start of the oxidation procedure (due to different extents of low level oxidation during isolation and storage) and also because of the well-known variability in the oxidisability of LDL between individual donors.

Some markers of oxidation are more useful than others, for instance, the lipid hydroperoxide levels in oxidised LDL increase, peak and then decrease to low levels (Fig. 7). If a batch of oxidised LDL is simply stated to have a lipid hydroperoxide content of say, 300 nmol/mg protein, this could either mean that the LDL is oxidised to only a relatively modest extent and the lipid hydroperoxides are still increasing or that it has been modified to a much greater extent and the lipid hydroperoxides are decreasing (Fig. 7). TBARS show a similar type of response, but the decrease in the TBARS after they have peaked is much slower. Other markers of oxidation increase and then remain relatively stable, but show little change during early stages of oxidation, for instance, the electrophoretic mobility or the rate of macrophage uptake.[51] There is, therefore, much to be said for measuring two or more markers of oxidation, one of which increases early during oxidation, for instance, lipid hydroperoxides or TBARS, and one which increases later in the oxidation process, for instance, the relative electrophoretic mobility. This would help different laboratories to compare the levels of oxidation of their LDLs more precisely, It should be borne in mind, however, that if LDL is oxidised by two different oxidative stresses to apparently the same extent as regards two markers of oxidation, it does not of course necessarily follow that other oxidation products will be present in similar amounts in the oxidised LDLs induced by different pro-oxidants. Different levels of oxidation of LDL will give it very different properties, and there may be "windows of oxidation" in which certain cellular functions are influenced by oxidised LDL, the effect not being seen with LDL with either higher or lower extents of oxidation. As the exact level of oxidation of LDL in atherosclerotic lesions is unknown (and no doubt varies between different types of lesions and even within different regions of the same lesion), there is a case for investigating the effects of LDL with a wide range of oxidation and for investigating LDL oxidised by a variety of different oxidants.

References

[1] D. Steinberg (1993) Modified forms of low-density lipoprotein and atherosclerosis. *Journal of Internal Medicine*, **233**, 227–232.

[2] C. Rice-Evans and K. R. Bruckdorfer (1995) "Oxidative Stress, Lipoproteins and Cardiovascular Dysfunction". Portland Press, London.

[3] H. N. Hodis, D. M. Kramsch, P. Avogaro, G. Bitolo-Bon, G. Cazzolata, J. Hwang, H. Peterson and A. Sevanian (1994) Biochemical and cytotoxic characteristics of an in vivo circulating oxidized low density lipoprotein (LDL-). *Journal of Lipid Research*, **35**, 669–677.

[4] S. Chirico, C. Smith, C. Marchant, M. Mitchinson and B. Halliwell (1993) Lipid peroxidation in hyperlipidaemic patients. A study of plasma using an HPLC-based thiobarbituric acid test. *Free Radical Research Communications*, **19**, 51–57.

[5] H. Esterbauer, M. Dieber-Rotheneder, G. Waeg, G. Striegl and G. Jurgens (1990) Biochemical, Structural and Functional Properties of oxidised Low-density lipoproteins. *Chemical Research in Toxicology*, **3**, 77–91.

[6] H. Puhl, G. Waeg and H. Esterbauer (1993) Methods to determine oxidation of low-density lipoproteins. *Methods in Enzymology*, **233**, 425–441.

[7] B. Halliwell and S. Chirico (1993) Lipid peroxidation: its mechanism, measurement and significance. *American Journal of Clinical Nutrition*, **57** (suppl), 715S–715S.

[8] B. H. Chung, T. Wilkinson, J. C. Geer and P. J. Segrest (1980) Preparative and quantitative isolation of plasma lipoproteins: rapid single discontinuous density ultracentrifugation in a vertical rotor. *Journal of Lipid Research*, **2**, 284–291.

[9] S. P. Gieseg and H. Esterbauer (1993) Low density lipoprotein is saturable by pro-oxidant copper. *FEBS Letters*, **343**, 188–194.

[10] D. F. Meyer, M. O. Mayans, P. H. E. Groot, K. E. Suckling, K. R. Bruckdorfer and S. J. Perkins (1995) Time-course studies by neutron solution scattering and biochemical assays of aggregation of human low-density lipoprotein during Cu^{2+}-induced oxidation. *Biochemical Journal*, **310**, 417–426.

[11] D. L. Tribble, L. G. Hall, P. D. Wood and R. M. Krauss (1992) Variations in oxidative susceptibility among six low density lipoprotein subfractions of differing density and particle size. *Atherosclerosis*, **93**, 189–199.

[12] S. C. Rumsey, N. F. Galeano, Y. Arad and R. J. Deckelbaum (1992) Cryopreservation with sucrose maintains

normal physical and biological properties of human plasma low density lipoproteins. *Journal of Lipid Research*, **33**, 1551–1561.

[13] J. A. Berliner, M. C. Territo, A. Sevanian, S. Ramin, J. Ai Kim, B. Bamshad, M. Esterson and A. M. Fogelman (1990) Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *Journal of Clinical Investigation*, **85**, 1260–1266.

[14] S. Parthasarathy, D. J. Printz, D. Boyd, L. Joy and D. Steinberg (1986) Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis*, **6**, 505–510.

[15] D. S. Leake and S. M. Rankin (1990) The oxidative modification of low density lipoproteins by macrophages. *Biochemical Journal*, **270**, 741–748.

[16] D. J. Lamb, G. M. Wilkins and D. S. Leake (1992) The oxidative modification of low density lipoprotein by human lymphocytes. *Atherosclerosis*, **92**, 187–192.

[17] J. Morgan, J. A. Smith, G. M. Wilkins and D. S. Leake (1993) Oxidation of low density lipoprotein by bovine and porcine aortic endothelial cells and porcine endocardial cells in culture. *Atherosclerosis*, **102**, 209–216.

[18] T. Henriksen, E. M. Mahoney and D. Steinberg (1981) Macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoprotein. *Proceedings of the National Academy of Sciences of the USA*, **78**, 6499–6503.

[19] T. Henriksen, E. M. Mahoney and D. Steinberg (1983) Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis*, **3**, 149–159.

[20] P. O'Brien (1969) Intracellular mechanisms for the decomposition of a lipid hydroperoxide I. Decomposition of a lipid peroxide by metal ions, haem compounds and nucleophiles. *Canadian Journal of Biochemistry*, **47**, 485–492.

[21] R. Labeque and L. Marnett (1988) Reaction of haematin with allylic fatty acid hydroperoxides. *Biochemistry*, **27**, 7060–7070.

[22] W. Sattler, D. Mohr and R. Stocker (1994) Rapid isolation of lipoproteins and assessment of their peroxidation by HPLC post column chemiluminescence. *Methods in Enzymology*, **233**, 469–489.

[23] H. Esterbauer, S. P. Gieseg, A. Giessauf, O. Ziouzenkova and P. Ramos (1995) Role of natural antioxidants in inhibiting Cu2+-mediated oxidation of LDL. In: Free Radicals, Lipoprotein Oxidation and Atherosclerosis (Bellomo G, Finardi G, Maggi, Rice-Evans C, eds) Richelieu Press, London pp. 11–26.

[24] C. Smith, M. J. Mitchinson, O. I. Aruoma and B. Halliwell (1992) Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochemical Journal*, **286**, 901–905.

[25] J. Morgan and D. S. Leake (1993) Acidic pH increases the oxidation of LDL by macrophages. *FEBS Letters*, **333**, 275–279.

[26] S. Parthasarathy (1987) Oxidation of low-density lipoprotein by thiol compounds leads to its recognition by the acetyl LDL receptor. *Biochimica et Biophysica Acta*, **917**, 337–340.

[27] J. W. Heinecke, H. Rosen, L. A. Suzuki and A. Chait (1987) The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *Journal of Biological Chemistry*, **262**, 10098–10103.

[28] G. M. Wilkins and D. S. Leake (1994a) The oxidation of low density lipoprotein by cells or iron is inhibited by zinc. *FEBS Letters*, **341**, 259–262.

[29] A. Graham, J. L. Wood, V. J. O'Leary and D. Stone (1994) Human (THP-1) macrophages oxidize LDL by a thiol-dependent mechanism. *Free Radical Research*, **21**, 295–308.

[30] J. L. Wood and A. Graham (1995) Structural requirements for oxidation of low density lipoprotein by thiols. *FEBS Letters*, **366**, 75–80.

[31] J. J. O. Turner, C. Rice-Evans, M. J. Davies and E. S. R. Newman (1991) The formation of free radicals by cardiac myocytes under oxidative stress and the effects of electron-donating drugs. *Biochemical Journal*, **277**, 833–837.

[32] G. Dee, C. Rice-Evans, S. Obeyesekera, S. Meraji, M. Jacobs and K. R. Bruckdorfer (1991) The modulation of ferryl myoglobin formation and its oxidative effects on low density lipoproteins by nitric oxide. *FEBS Letters*, **294**, 38–42.

[33] E. Newman, C. Rice-Evans and M. Davies (1991) Identification of initiating agents in myoglobin-induced lipid peroxidation. *Biochemical and Biophysical Research Communications*, **179**, 1414–1419.

[34] C. Rice-Evans, E. Green, G. Paganga, C. Cooper and J. Wrigglesworth (1993) Oxidised low density lipoproteins induce iron release from activated myoglobin. *FEBS Letters*, **326**, 177–182.

[35] N. Hogg, C. Rice-Evans, V. Darley-Usmar, M. T. Wilson, G. Paganga and L. Bourne (1994) The role of lipid hydroperoxides in the myoglobin dependent oxidation of LDL. *Archives of Biochemistry and Biophysics*, **344**, 39–44.

[36] C. Castelluccio, G. Paganga, N. Melikian, G. P. Bolwell, J. Pridham, J. Sampson and C. Rice-Evans (1995) Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants. *FEBS Letters*, **368**, 188–192.

[37] C. Castelluccio, P. Bolwell, C. Gerrish and C. Rice-Evans (1996) Differential distribution of ferulic acid to the major plasma constituents in relation to its potential as an antioxidant. *Biochemical Journal*.

[38] N. Salah, N. J. Miller, G. Paganga, L. Tijburg, G. P. Bolwell and C. Rice-Evans (1995) Polyphenolic flavanols as scavengers of aqueous phase radicals and chain-breaking antioxidants. *Archives of Biochemistry and Biophysics*, **322**, 339–346.

[39] C. Rice-Evans (1995) Plant polyphenols: free radical scavengers or chain-breaking antioxidants? In: Free Radicals and Oxidative Stress: environment, drugs and food additives (Rice-Evans C, Halliwell B, Lunt GG, eds) Portland Press, London.

[40] C. Rice-Evans, N. J. Miller and G. Paganga (1996) Structure-antioxidant activity relationships and phenolic acids. *Free Radical Biology and Medicine*, in the press.

[41] U. P. Steinbrecher, S. Parthasarathy, D. S. Leake, J. L. Witztum and D. Steinberg (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Sciences of the USA*, **81**, 3883–3887.

[42] W. Jessup, S. M. Rankin, C. V. de Whalley, J. R. S. Hoult, J. Scott and D. S. Leake (1990) α-Tocopherol consumption during low-density-lipoprotein oxidation. *Biochemical Journal*, **265**, 399–405.

[43] W. Jessup and R. T. Dean (1993) Autoinhibition of murine macrophage-mediated oxidation of low-density lipoprotein by nitric oxide synthesis. *Atherosclerosis*, **101**, 145–155.

[44] C. Mazière, M. Auclair and J-C. Mazière (1992) Lipophilic β-blockers inhibit monocyte and endothelial cell-mediated modification of low density lipoproteins. *Biochimica et Biophysica Acta*, **1126**, 314–318.
[45] D. J. Lamb and D. S. Leake (1994) Acidic pH enables caeruloplasmin to catalyse the modification of low density lipoprotein. *FEBS Letters*, **338**, 122–126.
[46] G. M. Wilkins and D. S. Leake (1994) The effect of inhibitors of free radical generating-enzymes on low density lipoprotein oxidation by macrophages. *Biochimica et Biophysica Acta*, **1211**, 69–78.
[47] Steinbrecher UP (1988) Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochimica et Biophysica Acta*, **959**, 20–30.
[48] K. Hiramatsu, H. Rosen, J. W. Heinecke, G. Wolfbauer and A. Chait (1987) Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis*, **7**, 55–60.
[49] M. K. Cathcart, A. K. McNally, D. W. Morel and G. M. Chisolm III (1989) Superoxide anion participation in human monocyte-mediated oxidation of low density lipoproteins and conversion of low-density lipoproteins to a cytotoxin. *Journal of Immunology*, **142**, 1963–1969.
[50] J. Morgan and D. S. Leake (1995) The oxidation of low density lipoprotein by iron or copper at acidic pH. *Journal of Lipid Research*, in press.
[51] K. L. H. Carpenter, G. M. Wilkins, B. Fussell, J. A. Ballantine, S. E. Taylor, M. J. Mitchinson and Leake D. S. (1994) Production of oxidized lipids during modification of low-density lipoprotein by macrophages or copper. *Biochemical Journal*, **304**, 625–633.
[52] E. B. Smith and E. M. Staples (1982) Plasma protein concentrations in interstitial fluid from human aortas. *Proceedings of the Royal Society of London, Series B*, **217**, 59–75.
[53] A. S. Khouw, S. Parthasarathy and J. L. Witztum (1993) Radioiodination of low density lipoprotein initiates lipid peroxidation: protection by use of antioxidants. *Journal of Lipid Research*, **34**, 1483–1496.
[54] E. Niki, N. Noguchi and N. Gotoh (1993) Dynamics of lipid peroxidation and its inhibition by antioxidants. *Biochemical Society Transactions*, **21**, 313–317.
[55] N. Noguchi, N. Gotoh and E. Niki (1993) Dynamics of the oxidation of low density lipoprotein induced by free radicals. *Biochimica et Biophysica Acta*, **1168**, 348–357.
[56] D. R. Arnelle and J. S. Stamler (1995) NO^+, NO and NO^- donation by S-nitrosothiols implications for regulation of physiological functions by S-nitrosylation and acceleration of disulphide function. *Archives of Biochemistry and Biophysics*, **318**, 279–285.
[57] J. Khan, D. M. Brennand, K. R. Bruckdorfer and M. Jacobs (1995) Nitrotyrosine-like immunoreactivity in human plasma of cigarette smokers and non-smokers. *British Journal of Pharmacology*, **116**, 328P.
[58] K. P. Moore, V. Darley-Usmar, J. Morrow and L. J. Roberts (1995) Formation of isoprostanes during oxidation of human low-density lipoproteins by peroxynitrite. *Circulation Research*, **77**, 335–341.
[59] J. Y. Hunt, R. T. Dean and S. P. Wolff (1988) Hydroxyl radical production and autoxidative glycosylation. *Biochemical Journal*, **256**, 205–212.
[60] H. Esterbauer, G. Striegl, H. Puhl and M. Rotheneder (1989) Continuous monitoring if in vitro oxidation of human low-density lipoprotein. *Free Radical Research Communications*, **6**, 67–75.
[61] C. Rice-Evans, G. Paganga and B. Andrews (1995) In vitro oxidation of low density lipoproteins as an index of oxidative risk. In: Free Radicals, Lipoprotein Oxidation and Atherosclerosis (Bellomo G, Finodi G, Maggi E, Rice-Evans C, eds) Richelieu Press, London, pp. 183–203.
[62] C. Rice-Evans, A. T. Diplock and M. C. R. Symons (1991) Techniques in Free Radical Research. Elsevier Science Publishers, Amsterdam.
[63] P. M. Abuja and H. Esterbauer (1995) Simulation of lipid peroxidation in low density lipoprotein by a basic "skeleton" of reactions. *Chemical Research in Toxicology*, **8**, 753–763.
[64] B. Vedie, I. Myara, M. A. Pech, J. C. Maziére, C. Maziére, A. Caprani and N. Moatti (1991) Fractionation of charge-modified low density lipoproteins by fast protein liquid chromatography. *Journal of Lipid Research*, **32**, 1359–1369.
[65] E. Hurt-Camejo, G. Camejo, B. Rosengren, F. López, C. Ahlström, G. Fager and G. Bondjers (1992) Effect of arterial proteoglycans and glycosaminoglycans on low density lipoprotein oxidation and its uptake by human macrophages and arterial smooth muscle cells. *Arteriosclerosis and Thrombosis*, **12**, 569–583.
[66] Louise Bourne (1996) PhD thesis, University of London.
[67] L. G. Fong, S. Parthasarathy, J. L. Witztum and D. Steinberg (1987) Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *Journal of Lipid Research*, **28**, 1466–1477.
[68] G. Jürgens, H. F. Hoff, G. M. Chisolm III and H. Esterbauer (1987) Modification of human serum low density lipoprotein by oxidation—characterization and pathophysiological implications. *Chemistry and Physics of Lipids*, **45**, 315–336.
[69] L. Cominacini, U. Garbin, A. Davoli, R. Micciolo, O. Bosello, G. Gaviraghi, L. A. Scuro and A. M. Pastorino (1991) A simple test for predisposition to LDL oxidation based on the fluorescence development during copper-catalyzed oxidative modification. *Journal of Lipid Research*, **32**, 349–358.
[70] J. Nourooz-Zadeh, J. Tajaddini-Sarmadi and S. P. Wolff (1994) Measurement of Plasma Hydroperoxide Concentrations by the Ferrous Oxidation-Xylenol Orange Assay in Conjunction with Triphenyl Phosphine. *Analytical Biochemistry*, **220**, 403–409.
[71] Z. Jiang, J. V. Hunt and S. P. Wolff (1992) Ferrous Ion Oxidation in the Presence of Xylenol Orange for Detection of Lipid Hydroperoxide in Low Density Lipoprotein. *Analytical Biochemistry*, **202**, 384–389.
[72] M. El-Saadani, H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar and G. Jürgens (1989) A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *Journal of Lipid Research*, **30**, 627–630.
[73] H. Esterbauer, J. Gebicki, H. Puhl and J. Jurgens (1992) The role of lipid peroxides and antioxidants in the oxidative modification of LDL. *Free Radical Biology and Medicine*, **13**, 341–390.
[74] V. O'Leary, V. Darley-Usmar, L. Russell and D. Stone (1992) Pro-oxidant effects of lipoxygenase-derived peroxides on the copper-initiated oxidation of low density lipoprotein. *Biochemical Journal*, **282**, 631–634.
[75] L. Kritharides, W. Jessup, J. Gifford and R. T. Dean (1995) A method for defining the stages of oxidation by the separation of cholesterol and cholesterol ester oxidation products using HPLC. *Analytical Biochemistry*, **213**, 79–89.

[76] R. Stocker, V. Bowry and B. Frei (1991) Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha tocopherol. *Proceedings of the National Academy of Sciences*, **88**, 1646–1650.
[77] K. P. Moore, V. Darley-Usmar, J. Morrow and L. J. Roberts (1995) Formation of F_2-isoprostanes during oxidation of human low density lipoprotein and plasma by peroxynitrite. *Circulation Research*, **77**, 335–341.
[78] V. C. Reid and M. J. Mitchinson (1993) Toxicity of oxidised low density lipoprotein towards mouse peritoneal macrophages in vitro. *Atherosclerosis*, **98**, 17–24.
[79] Z. A. Cohn and B. Benson (1965) The differentiation of mononuclear phagocytes. Morphology, cytochemistry and biochemistry. *Journal of Experimental Medicine*, **121**, 153–170.
[80] S. E. Stait and D. S. Leake (1994) Ascorbic acid can either increase or decrease low density lipoprotein modification. *FEBS Letters*, **341**, 263–267.
[81] I. Jialal, G. L. Vega and S. M. Grundy (1990) Physiologic levels of ascorbate inhibit the oxidative modification of low density lipoprotein. *Atherosclerosis*, **82**, 185–191.
[82] B. L. Knight and A. K. Soutar (1982) Changes in the metabolism of modified and unmodified low density lipoproteins during the maturation of cultured blood monocyte-macrophages from normal and homozygous familial hypercholesterolaemic subjects. *European Journal of Biochemistry*, **125**, 407–413.
[83] C. A. Drevon, A. D. Attie, S. H. Pangburn and D. Steinberg (1981) Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *Journal of Lipid Research*, **22**, 37–46.
[84] M. E. Knowles and D. S. Leake (1989) The effects of acetylated low density lipoproteins on fluid-phase pinocytosis by macrophages. *European Journal of Biochemistry*, **182**, 407–412.
[85] M. Lougheed, H. F. Zhang and U. P. Steinbrecher (1991) Oxidized low density lipoprotein is resistant to cathepsins and accumulates within lysosomes. *Journal of Biological Chemistry*, **266**, 14519–14525.
[86] S. M. Rankin, M. E. Knowles and D. S. Leake (1988) Macrophage protease activity towards native and modified low density lipoproteins. In Hyperlipidaemia and Atherosclerosis (K. E. Suckling and P. H. E. Groot, eds.), pp. 214–215, Academic Press, London.
[87] E. L. Mander, R. T. Dean, K. K. Stanley and W. Jessup (1994) Apolipoprotein-B of oxidized LDL accumulates in the lysosomes of macrophages. *Biochimica et Biophysica Acta*, **1212**, 80–92.
[88] D. J. Lamb and D. S. Leake (1992) The effect of EDTA on the oxidation of low density lipoprotein. *Atherosclerosis*, **94**, 35–42.
[89] J. C. Khoo, E. Miller, P. McLoughlin and D. Steinberg (1988) Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis*, **8**, 348–358.