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Review

Enzymic mechanisms of superoxide production

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Contents

Abbreviations: O_2^-, superoxide; XDH, xanthine dehydrogenase; XO, xanthine oxidase; EBV, Epstein-Barr virus; CGD, chronic granulomatous disease.

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I. Introduction

The realization that superoxide, and other reactive oxygen species, is produced in significant quantities in biological systems is a relatively recent one and the nature and purpose of the radical-generating systems is in most cases, only beginning to be understood. It is the purpose of this review to describe the known biological sources of superoxide, the enzymic machinery used to generate it, and the possible functions to which it is put.

II. Free radicals derived from autoxidation of small molecules

There are a number of small intra- and extracellular components which are capable of undergoing redox reactions with molecular oxygen to generate superoxide (O_2^-). These include thiols [1], flavins [2,3], quinones, catecholamines [4] and pterins [5,6]. In addition many xenobiotics exert their biological effects, both beneficial and detrimental, through the production of O_2^- and other reactive oxygen species by undergoing redox cycling reactions with enzymes, notably NADPH–cytochrome *P*-450 reductase [7].

III. Superoxide as an enzyme-bound intermediate

Superoxide is an enzyme bound intermediate in a number of enzymic reactions and can be bound to a variety of prosthetic groups: flavins (in 2-nitropropane dioxygenase [8]); haem (in cytochrome *P*-450 [9]); copper (in galactose oxidase [10]); non-haem iron (in ferredoxin [11]). The bound superoxide is not normally released. In addition to superoxide dismutase, at least one enzyme, indolamine 2,3-dioxygenase uses O_2^- as a substrate [12].

IV. Neutrophil NADPH oxidase

IV-A. Composition and properties

The superoxide generating system of neutrophils is a membrane-associated enzyme complex present in a dormant form in unstimulated cells which is converted to the active form after treatment of the cells with a suitable agonist. This is accompanied by the consumption of oxygen (the respiratory burst) and the production of the superoxide anion radical. This "increased respiration of phagocytosis" was first reported by Baldridge and Gerrard in 1933 [13] and was thought to result from the stimulation of mitochondrial respiration to provide energy for phagocytosis. The system responsible for the respiratory burst was shown to be non-mitochondrial in origin when it was found to be insensitive to the classic mitochondrial inhibitors, cyanide and antimycin [14]. The same oxidase is believed to be present in all the microbicidal phagocytic cells; polymorphonuclear leukocytes (PMN, neutrophils); monocytes; macrophages (including at least some tissue macrophages) and eosinophils. It consists of at least two redox active components, a flavin adenine dinucleotide (FAD)-containing flavoprotein and a unique low-potential *b*-type cytochrome, cytochrome *b*-245. It has been the subject of a number of recent excellent reviews

[15–17]. The source of electrons for the reduction of oxygen is NADPH, formed by the hexose monophosphate pathway; the activity of the latter is greatly stimulated during the respiratory burst. The flavoprotein is likely to accept two electrons from NADPH and pass them one at a time to cytochrome *b*-245, which in turn performs a single electron reduction of molecular oxygen to form O_2^-, as shown in Scheme I.

$$\text{NADPH} \xrightarrow{2e^-} \text{Flavoprotein} \xrightarrow{e^-} \text{Cytochrome } b\text{-}245 \xrightarrow{e^-} O_2 \rightarrow O_2^-$$

Scheme I.

Cytochrome *b*-245 was originally reported in equine neutrophils by Hattori in 1961 [18] and it was later suggested by Shinagawa [19] that it might be involved in the respiratory burst on the basis of its autoxidizability and capacity to bind carbon monoxide, both properties expected of cytochrome oxidases. This work was rather ignored until the observations of Segal and co-workers that the cytochrome was absent from the neutrophils of some [20,21] but not all [22] patients with chronic granulomatous disease (CGD) in which the respiratory burst is absent. The cytochrome was found to be localized in both the plasma membrane and the specific granule fraction. The cytochrome became incorporated into the phagolysozomal membrane during phagocytosis [23,24] and became reduced on stimulation of the cells under anaerobic conditions and reoxidized when air was readmitted [25]. In those CGD patients who had the cytochrome it was not reduced on stimulation, suggesting the deficiency of some other component [26]. These findings were elegantly supported by complementation studies in monocytes using cells from patients with cytochrome *b*-245-positive and -negative forms of CGD [27]. Recent work has shown that the majority of patients with the cytochrome *b*-positive form of CGD are lacking one or other of the two cytosolic proteins of molecular mass 47 kDa or 66 kDa which appear to be involved in the activation process (reviewed by Curnutte, Ref. 28). The cytochrome has an extremely low midpoint oxidation-reduction potential ($E_{m7} = -245$ mV) [29], lower than any other mammalian cytochrome and sufficiently low to reduce oxygen to superoxide ($E_{m7} = -160$ mV [30]). The cytochrome was originally detected only in the specialized phagocytic cells [31] (but vide infra); its synthesis was induced in both the DMSO-induced differentiation of the promyelocytic HL-60 cell line and after interferon-τ treatment of a macrophage cell line, concomitantly with the ability to produce O_2^- [32,33]. In common with many terminal oxidases, cytochrome *b*-245 can form complexes with ligands other than molecular oxygen, but with rather low affinities [19,34–43]. The K_m of the oxidase for oxygen is between 5 and 30 μM [42,44–46] but for the classic cytochrome oxidase inhibitor, carbon monoxide, the K_m is much higher (1.4 mM) and CO is not inhibitory in the presence of oxygen [34]. Such resistance to CO is not unique among oxygen-ligating haemoproteins. Decreased affinity can be due to steric hindrance of the linear Fe-CO complex by amino-acid residues near the binding site which favour the bent Fe-O$\backslash_{O}$ ligand [47,48]. Hurst and Barrette have suggested that cytochrome *b*-245 shows chemical properties consistent with those of a strongly hydrogen-bonded mono- or bis-histidyl hemin, which would also be compatible with reduced CO-binding affinities [15]. The cytochrome also binds pyridine, imidazole and butyl isocyanide, which are inhibitors of the oxidase [49,50]. Kinetic studies have shown the reduced cytochrome to be reoxidized by oxygen very rapidly (vide infra) [34,51–53]. In most purifications of the oxidase the cytochrome *b*-245 has been found to co-purify with enzymic activity [35,36, 38,50,51,54–60], although this has not always been the case [61–63]. Cytochrome *b*-245 has now been purified and shown to be a heterodimeric protein consisting of a heavily glycosylated (β) subunit of 76–92 kDa and non-glycosylated 23 kDa (α) subunit [64–66], both of which are absent in the more common form of X-linked CGD. Both genes have been sequenced [67,68] but the derived amino acid sequences bear no obvious similarity to those of other cytochromes, with the exception of a limited region in the α-subunit which is similar to a region of mitochondrial cytochrome oxidase polypeptide I [68] and to bovine chromaffin granule cytochrome *b* [69]. Two recent studies suggest the haem-bearing subunit is the α subunit [43,70]. Surprisingly, the α-subunit appears to be transcribed in many non-myeloid cell types in the absence of the haem spectrum [68] and is not regulated by interferon-τ [71], unlike the β subunit which is largely confined to myeloid cells and is upregulated by interferon.

The involvement of a flavoprotein had been suggested from the early studies of Babior and co-workers, who found that the addition of FAD to detergent extracts of oxidase containing membranes restored activity lost in the presence of certain detergents [59,72,73]. Enzymic activity was inhibited by certain flavin analogues whilst the oxidase activity in the presence of other flavin analogues correlated with their oxidation-reduction potential [51,74]. The subcellular distribution of FAD is found to be similar to that of cytochrome *b*-245 [75] and there are altered levels of FAD in the neutrophils of patients with CGD [75–78]. Light-induced photoreduction of flavin results in the reduction of cytochrome *b*-245 in the absence of oxygen [42,75] and in oxygen uptake under aerobic conditions (Cross and Jones, unpublished data). In the presence of NADPH both cytochrome and flavoprotein(s) are reduced under anaerobic conditions [50,52]. FAD has been found in the most purified preparations of the oxidase [55,59,62] although in differing amounts, per-

haps reflecting a progressive loss during purification procedures and the difficulty in its accurate estimation in small quantities of pure enzyme. The flavoprotein is fluorescent with excitation and emission bands similar to those of free FAD [52,75,79,80]. The redox potential of the flavin has been determined by ESR spectroscopy to be −280 mV [46] and is thus thermodynamically competent to participate in electron transfer from NADPH ($E_{m7} = -320$ mV) to cytochrome *b*-245 ($E_{m7} = -245$ mV). The redox potential was the same whether the membranes were prepared from resting or active cells. The flavin semiquinone was formed much more rapidly in the presence of NADPH in preparations of active membranes compared to those made from resting cells, suggesting little interaction between NADPH and FAD in the resting enzyme. The semiquinone formed is of the blue neutral type [46,74], commonly found in flavoproteins of the dehydrogenase/electron transferase type, such as cytochrome *P*-450 reductase [81]. Partial purification of the flavoprotein has resulted in preparations where the major component polypeptide had a molecular mass of 51 kDa (by gel filtration [57]), 72 kDa, 87 kDa (both by non-denaturing gel electrophoresis [82,83]) or 65–67 kDa (by SDS-PAGE [62,63,84,85]). Affinity labelling with reactive NADPH analogues have also identified proteins of 65–68 kDa [86,87] and 45, 55 and 66–70 kDa [85]. It has recently been shown that the 66 kDa membrane component labelled with *o*-dialdehyde NADPH is probably not part of the oxidase [85,88]. We have used the potent inhibitor of the neutrophil superoxide-generating system, diphenylene iodonium (DPI),to label a single polypeptide of 45 kDa (by SDS-PAGE) in neutrophils [89], macrophages [90] and eosinophils [91]. This polypeptide is induced during interferon-τ treatment of a monocytic cell-line in synchrony with the ability to produce O_2^- [33]. The polypeptide has been purified and has properties consistent with it being the flavoprotein component of the oxidase [80].

Some workers have suggested that ubiquinone may be a component in the electron transport pathway of the radical generating system [58,92–96]. However, analyses by other groups have found only small quantities of ubiquinone in neutrophils, and this has been associated with the mitochondrial fractions [97]. Ubiquinone is not present in cytoplasts, which can perform a normal respiratory burst [98], or in partially purified oxidase preparations [55,62] and its participation in electron transfer reactions within the oxidase is unlikely on thermodynamic grounds.

An important advance in the understanding of the superoxide-generating system was made with the development of a cell-free activation system consisting of disrupted resting cells, NADPH and SDS or arachidonate [99–102]. Fractionation of membranes, membrane extracts and cytosol fractions has identified several components in these compartments [68,102–106]. By mixing cytosol and membranes from normal individuals and patients with various types of CGD it has been possible to localize the sites of the patients' lesions. Two cytosolic proteins of 47 kDa and 65–67 kDa have been identified which are essential for oxidase activity and which are missing in the commoner forms of autosomal recessive CGD [107–112]. Sequence data suggest that these proteins are GTP-binding proteins related to the *ras* and *src* families. A further *ras*-related 22 kDa protein has been shown to associate with the cytochrome *b*-245 [113]. Addition of GTP or its non-hydrolysable analogues markedly increases oxidase activity in the cell-free system [105,114–120]. In addition to these two proteins, both data obtained by the fractionation of cytosol and data obtained from experiments measuring the kinetic of activation of the oxidase indicate that there are likely to be at least two more cytosolic factors required, but these are so far unidentified [106,111,121–123].

IV-B. Electron transfer mechanism in NADPH oxidase

Measurements on NADPH oxidase solubilized from activated neutrophil membranes show that the electrons donated by NADPH are quantitatively transferred to O_2 forming stoichiometric amounts of O_2^- (Cross, unpublished observations); no H_2O_2 is formed directly by the oxidase and the reaction may be represented thus:

$$NADPH + H^+ + 2O_2 \xrightarrow{\text{oxidase}} NADP^+ + 2H^+ + 2O_2^- \quad (1)$$

The pK of the dissociation:

$$O_2H \rightleftarrows O_2^- + H^+ \quad (2)$$

is 4.7 [124] and so superoxide is present very largely as the anion at physiological pH. Wood [30] has calculated that the couple O_2/O_2^- has $E_0 = -330$ mV (at $p_{O_2} = 1$ atm). Even under non-standard conditions, of equal concentrations of dissolved O_2 and O_2^-, the E_{m7} for O_2/O_2^- is −160 mV. Thus for kinetic efficiency the direct donor of electrons from the oxidase complex to O_2 should have E_{m7} close to, or lower than, −160 mV. The arrangement of electron transfer components suggested in Scheme I is compatible with this requirement. The abundant cytochrome *b* which is characteristic of neutrophils and which is present in other 'professional phagocytes' has $E_{m7} = -245$ mV [29].

When dithionite-reduced neutrophil plasma membranes or solubilized oxidase were rapidly mixed with oxygenated buffers the cytochrome *b* was oxidized quickly [53], with a pseudo-first-order rate constant of 147 s^{-1}. The addition of NADPH to active oxidase caused approx. 8.8% reduction of the cytochrome *b* in aerobic steady-state spectra, indicating that the turnover

of the cytochrome *b* was 13.08 mol s^{-1}. Since the measured rate of superoxide production by this preparation was 13.03 mol s^{-1} (mol cytochrome *b*)$^{-1}$ it can be seen that all the electrons transferred to O_2 by the NADPH oxidase passed through the cytochrome *b*. Thus, it is extremely unlikely that $FADH_2$ or flavin semiquinone ($FADH^{\cdot}$) formed during oxidase turnover interacts directly with O_2 to make O_2^-.

The oxidation-reduction properties of the FAD associated with plasma membrane NADPH oxidase have been determined by ESR spectroscopy [46]. In both stimulated and resting cells the E_{m7} of the flavin was −280 mV. A flavin free-radical intermediate was detected on addition of NADPH, with an ESR signal suggesting that it was a neutral semiquinone; this signal was absent from membranes prepared from unstimulated cells. The flavin free radical titrates with E_{m7} for the FAD/$FADH^{\cdot}$ couple of −304 mV and E_{m7} for $FADH^{\cdot}/FADH_2$ of −256 mV. These midpoint potentials are sufficiently low that plasma membrane reduced flavin could reduce O_2 directly to form O_2^-, but the kinetic studies quoted above suggest that this is unlikely, and that the FAD participates in two electron transfer from NADPH and one electron transfer to cytochrome *b*. A possible arrangement for such transfers is shown in Scheme II. Each of the flavin semiquinone couples is sufficiently reducing to reduce the cytochrome *b* with its $E_{m7} = -245$ mV.

During steady electron transfer from NADPH to O_2 the cytochrome *b* of the oxidase is typically around 10% reduced and the FAD 53% reduced [53,125]. From the Nernst equation it can be calculated that the cytochrome *b* is poised at −190 mV. At this redox poise the FAD should be only 0.09% reduced. Obviously, there is a barrier to the transfer of electrons from flavin to cytochrome *b*, resulting in extensive flavin reduction. Visible spectroscopy gives no evidence for the presence of a flavin semiquinone: the latter may be unstable, perhaps undergoing disproportionation to restore $FADH_2$, and this suggestion is supported by the measurements [46] showing that E_{m7} FAD/$FADH^{\cdot}$ is more reducing than E_{m7} $FADH^{\cdot}/FADH_2$, unlike most pyridine nucleotide-dependent flavoproteins. The kinetic impediment to the transfer of electrons between FAD and cytochrome *b* may arise from the process of activation. In 'resting' oxidase the flavoprotein does not receive electrons from NADPH [50]; activation exposes the flavin to reduction. It is at present unclear whether all the membrane flavoprotein undergoes activation and whether the activation process involves any change in the association of flavoprotein and cytochrome *b*.

Results from experiments using inhibitors support a form of organization of components of the NADPH oxidase where electrons flow from FAD to cytochrome *b* (Scheme I). The haem ligand imidazole inhibits NADPH oxidase: it inhibits reduction of the cytochrome *b* but permits reduction of FAD. The oxidase inhibitor [89,126] diphenylene iodonium (DPI), which binds to the flavoprotein component [127] prevents reduction of both FAD and cytochrome *b* by added NADPH.

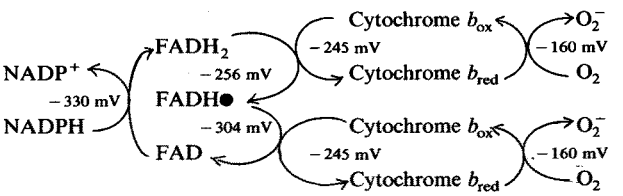
IV-C. Electrogenic nature of NADPH oxidase

The O_2^- produced by activated phagocytes is released on the outer face of the cell, where it enters the developing phagocytic vacuole and contributes to the microbicidal activity of phagocytes. The electron donor to the oxidase is NADPH produced in the cytosol by the pentose phosphate pathway (Scheme III). If the transfer of electrons is not accompanied by movement of other ions its activity will be electrogenic.

A number of papers have shown that activation of neutrophils and other phagocytes is associated with changes of the plasma membrane potential. Effects on the transmembrane potential charge have usually been determined from stimulus-induced changes in the fluorescence of cationic cyanine dyes, such as di-O-C_5, di-O-C_6 and di-O-C_3 or from absorbance changes of the anionic oxonols, e.g., Oxonol V. Measurements of membrane potentials of whole neutrophils are complicated by the presence of large numbers of membrane-limited granules in the cytoplasm which contain proteins involved in the microbicidal functions of the leukocytes. The distribution of added lipophilic anionic or cationic probes of membrane potential across the membranes of these intracellular compartments may complicate interpretation of the absorbance or fluorescence changes. This may account for a lack of consistency in the reported effects of neutrophil activation on membrane potential.

Most authors agree that activation leads to a depolarisation of the plasma membrane although there have been reports of a hyperpolarization preceding the onset of superoxide generation [128–130]. Most workers report a depolarization of the membrane on activation of phagocytes [131–135], but it was suggested that depolarization preceded superoxide production and that this change was part of receptor-response coupling, perhaps due to an influx of Ca^{2+} or Na^+.

It is possible to prepare cytoplasts from neutrophils; these are vesicles of cytosol enclosed in plasma membrane but lacking the intracellular organelles [136]. Cy-



Scheme II. Possible redox couples involved in the transfer of electrons in NADPH oxidase.

toplasts retain the capacity to produce O_2^- when treated with activators and have the same plasma membrane potential as intact neutrophils. When loaded with indicators of membrane potential the results are not distorted by partition of the probe into granule or mitochondrial compartments and the low peroxidase content of cytoplasts diminishes the risk of peroxidative attack on the probe. Using Oxonol V as a probe, it was found [137] that the soluble stimulus, phorbol myristate acetate (PMA), caused a depolarization of the plasma membrane by about 30 mV, developing over about 1 min. The depolarization coincided with the onset of O_2^- generation, and a new stable value for the membrane potential of about -30 mV was attained, whilst O_2^- production continued almost linearly. When NADPH oxidase was inhibited by the addition of DPI [89,126], superoxide generation by cytoplasts ceased and the plasma membrane began to repolarize. Similarly, the depolarization of PMA-activated cytoplasts was much diminished when DPI was present. These effects are best explained by suggesting that NADPH oxidase is electrogenic and so depolarizes the plasma membrane, but that in order to maintain electron flow and a stable membrane potential a channel opens which permits the movement of a counter ion. This conclusion is supported by the early observation of Seligmann and Gallin [133] that the membrane potential of neutrophils from CGD patients did not depolarize when they were stimulated with PMA or fMet-Leu-Phe. Such CGD neutrophils respond normally with respect to degranulation and chemotaxis.

The cytosol of neutrophils or cytoplasts becomes more acid following PMA stimulation and this has been extensively studied by Henderson and Grinstein and their collaborators. The major contributor to this increased $[H^+]$ is likely to be the NADPH oxidase itself, which produces $1H^+/e^-$ transferred to O_2, as shown below (Scheme III).

The maximum acidification of activated neutrophils measured using the fluorescent pH indicator BCECF is seen only when the Na^+/H^+ channel is blocked by amiloride and Cd^{2+} or Zn^{2+} ions are added to block an H^+ channel [138,139]. Under these conditions neutrophil cytoplasts retained $0.8H^+/O_2^-$ generated; when the inhibitors were absent the intracellular H^+ increase was much diminished. We have suggested [138] that the results are best explained if the NADPH oxidase is electrogenic and that H^+ arising from NADPH oxidation cross the membrane via a Cd^{2+} or Zn^{2+}-sensitive proton conducting channel (Scheme III).

Thus H^+ acts as a compensating ion to accompany the e^- moved electrogenically to reduce O_2 to O_2^-.

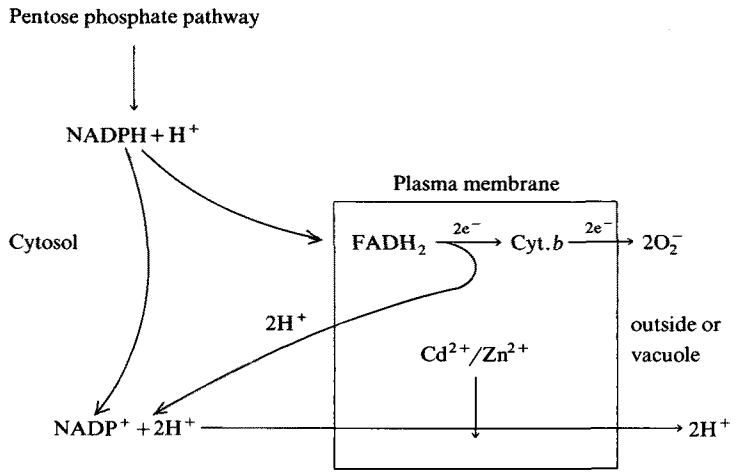
The rapid spontaneous dismutation of the superoxide anion consumes H^+:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad (3)$$

Consequently, there is little acidification of the external medium when neutrophil NADPH oxidase is activated. A slight decrease in pH is observed which probably derives from the CO_2 arising from the hexose monophosphate pathway in generating NADPH for the oxidase.

In CGD neutrophils [140] there was no cytosolic acidification following treatment with PMA. Indeed these cells showed significant alkalinization, which was amiloride sensitive suggesting that Na^+/H^+ exchange was activated by the PMA. Similarly, Henderson et al. [138] showed that cytoplasmic acidification was prevented in cells treated with DPI to inhibit NADPH oxidase.

In neutrophil cytoplasts suspended in a high-K^+ medium, the addition of valinomycin makes the membrane potential positive. When the NADPH oxidase is activated it is now energetically more difficult to translocate e^- electrogenically to make O_2^-. Under these conditions the addition of an uncoupler such as FCCP makes possible the ready transfer of intracellular H^+ to



Scheme III. Relationship of electron transfer by NADPH oxidase and transfer of H^+ via a channel.

accompany the e^- and the formation of O_2^- is stimulated – a special variety of respiratory control [141].

The nature of the H^+ channel is at present unclear: it could be a component of the NADPH oxidase itself. These is no decisive evidence concerning its regulation, although a voltage gating system is attractive.

IV-D Inhibition of NADPH oxidase

There is a large number of compounds described in the literature as inhibitors of the O_2^--generating system of phagocytes and the subject has been recently been reviewed [142]. The phagocyte responds to a wide range of different stimuli, which may involve a number of different signalling mechanisms, and the oxidase itself is complex, all of which presents a number of different target sites for inhibitors. The inhibitors can be broadly divided into two groups, those which inhibit activation of the oxidase and those which exert a direct inhibitory effect. As a result of the complexity of the signalling system there are a large number of more or less specific inhibitors which interact with these different signal transduction pathways. Many of these are known to be inhibitors of particular second messenger systems, and their effects are attributed to the known inhibitory properties. Unfortunately, it is clear that many of these agents are capable of interacting with the oxidase in ways which are quite independent of their recognised mechanisms and their description as specific inhibitors must be treated with caution [142–144]. Compounds which affect the activation pathways may do so in a number of ways, they can interfere with ligand binding or receptor expression [145–155], signal transduction [156–158], second messenger formation [150,159–165], activation of protein kinases [166–170], assembly of oxidase components [171] or they may interfere in the generation of NADPH required as the substrate by either inhibition of the hexose monophosphate shunt [172] or glucose uptake [173]. In addition they may increase the rate of deactivation of the oxidase [174]. Direct acting inhibitors can affect the flavoprotein [50,51,60,85,87–89,126,142,175,176], cytochrome *b*-245 [49,50], essential phospholipid environment [50,176], divalent ion requirement [177,178], essential thiols [36,50, 60,176,179] or may inhibit charge translocation [142]. In addition to those compounds whose site of action is known or inferred, there are a number of compounds which do not have obvious targets [142].

V. O_2^- production by non-phagocytes

Many cell-types and tissues have the capacity to form O_2^-, often without any obvious function but sometimes in response to specific stimuli. An account of these systems is given below.

V-A. Phagocyte oxidase-like

V-A.1. Lymphocytes

Human B-lymphocytes which have been transformed by treatment with Epstein-Barr virus express a superoxide generating system [180] which is similar in many respects to that of neutrophils. Indeed, EBV-B-lymphocytes from CGD patients resemble the neutrophils from such patients in their absence of superoxide generating oxidase. The transformed B-lymphocytes contain the low-potential cytochrome *b* and the DPI-binding 45 kDa protein characteristic of the NADPH oxidase of phagocytes [181], although the K_m for NADPH and for NADH were different [182].

The oxidase of EBV-B lymphocytes was activated not only by PMA but also by protein-A-bearing staphylococci and by mouse monoclonal antibodies to human IgG in the presence of a second cross-linking anti-mouse-Ig antibody. Some sort of stabilization of surface immunoglobulin aggregate appears necessary for effective signal transduction [183]. The oxidase of EBV-transformed B-lymphocytes can also be stimulated by the cytokines TNF α and IL-1β and bacterial lipopolysaccharides [184], which suggests that superoxide release may be a normal function of B-lymphocytes, related perhaps to proliferation and differentiation of these cells.

The EBV-B lymphocyte oxidase can be activated by treating whole cells with Ca^{2+}-ionophores, AlF_3 and arachidonate [184] suggesting that its activation pathway is similar to that of phagocyte NADPH oxidase.

It is noteworthy that the low-potential cytochrome *b*, characteristic of the NADPH oxidase, can be detected in normal peripheral B lymphocytes [183]. It is not expressed at early stages of the differentiation of B lymphocytes (in pre-pre B cells or pre B cells), but appears at the early B cell stage [185]. It disappears from the B cell surface during final differentiation to plasma cells. This transient expression of the cytochrome suggests that the superoxide generation may be important for B lymphocyte function at certain stages of development.

V-A.2. Fibroblasts

Fibroblasts have recently been reported to release O_2^-. Murrell et al., reported a rate of release similar to that of resting (unstimulated) neutrophils [186] which was doubled after treatment of the cells with PMA and Ca^{2+} (compared to a 20–30-fold increase for PMN). Meier et al. [187] reported no detectable O_2^- production from adherent fibroblasts, but showed a dose-dependent stimulation of O_2^- in response to interleukin-1α or tumour necrosis factor-α (TNF) up to approx. 5 nmol O_2^-/h per 10^6 cells which continued for 4 h. TNF and other cytokines have also been shown to elicit O_2^- from adherent neutrophils, macrophages, endothelial cells and

EBV-transformed B-lymphocytes [184,188–191]. The source of O_2^- was not XO, as it was not inhibited by allopurinol, or mitochondrial, as the system was insensitive to cyanide, azide and rotenone. Interestingly, the basal rate of O_2^- release by fibroblasts reported by Murrell was the same as that which was shown to stimulate fibroblast proliferation, suggesting a role in the inflammatory process [192]. Fibroblasts are known to participate in joint-damaging processes in rheumatoid arthritis; the release of O_2^- might cause a direct damaging effect and also could function in the regulation of lymphocyte activity.

V-A.3. Endothelial cells

Endothelial cells release O_2^- at low rates (9.15 nmol/h per 10^6 cells), treatment of the cells with the cytokines interferon-τ and interleukin-1 increased this radical release (to 27.6 nmol/h per 10^6 cells). The O_2^- produced by the endothelial cells may participate in vascular and connective tissue injury [190]. IL-1 accelerates the proliferation of endothelial cells [193], and in view of the recently described effect of O_2^- on fibroblast proliferation (above) it would be of interest to see if endothelial cells behave in a similar manner. A recent report describes the conversion of XDH to XO in endothelial cells in response to inflammatory mediators (C5a, TNF or fMLP) [194], although bovine aorta endothelial cells do not contain XO and therefore cannot be the source of O_2^- in this tissue [195]. The resultant generation of O_2^- can cause the recruitment of neutrophils by the generation of chemotactic lipids [196,197], and activated neutrophils can also cause the conversion of XDH to XO in endothelial cells [198], which results in endothelial cell death. Endothelium derived vascular contraction factor ($EDCF_2$) has been suggested to be O_2^- [199], supporting the suggestion of O_2^- acting as a messenger molecule in some tissues [200].

V-A.4. Mesangial cells

Kidney glomeruli, and the mesangial cells which are present in them, have been shown to produce O_2^-. Glomeruli depleted of leukocytes have been reported to produce SOD-inhibitable chemiluminescence in response to PMA which shows a lag phase similar to that seen in phagocytes [201]. This activity could also be induced by serine proteinases which can also stimulate neutrophils [202]. As in neutrophils, the activity is inhibited by sulphydryl reagents, but unlike them, it is also inhibited by antimycin. Cultured rat mesangial cells have been reported as producing high levels of O_2^- (28–68 nmol O_2^-/10 min per mg) in response to complement components [203] or opsonized zymosan [204], the latter activity can be inhibited by histamine [205] probably by inducing an increase in cAMP [206]. The opsonized zymosan-induced O_2^- was also sensitive to pretreatment of the cells with dexamethazone, an agent which inhibits neutrophil responses to fMLP [151]. The recent finding that high levels of the mRNA coding for the small subunit of the neutrophil cytochrome *b*-245 are present in mouse kidney [207] may indicate the same O_2^- producing system is present in these cells.

VI. Reduced oxygen species from organ systems

VI-A. Reproductive tissue

VI-A.1. Spermatazoa

Several authors have reported the production of reduced oxygen species from spermatozoa [208,209]. Aitken and Clarkson [210] suggested that O_2^- is the product after stimulation of spermatozoa with the calcium ionophore A23187. The activity was not inhibited by mitochondrial inhibitors and NADPH was the probable donor, as its addition to permeabilized sperm stimulated O_2^- production. The authors speculate that the system might be involved in raising intracellular pH, which is associated with cellular activation. In another study, O_2^- production from spermatozoa has been inversely related to sperm motility. The electron donor for the reaction was not identified [211].

VI-A.2. Leydig cells

There is a preliminary report of the production of O_2^- in rat testis Leydig cells which appears to be stimulated by luteinizing hormone [212]. The activity was suggested to be intracellular, in contrast to most other systems, and required the inhibition of intracellular SOD in order to detect O_2^- formation. It was proposed the activity might be involved in the generation of lipid peroxides which are thought to be necessary for the activation of lipoxygenase/cyclooxygenase [213,214].

VI-A.3. Uterus

A recent paper has described the formation of O_2^- by mouse ovary and uterus which is suggested to play a role in implantation [215]. It is not clear if this radical production is related to the earlier fertilization events which, in sea-urchin eggs, are known to involve oxygen radical production (described below).

VI-B. Other organ systems

VI-B.1. Thyroid

There is a system in the thyroid gland which is necessary for the production of H_2O_2 for thyroid hormone biosynthesis via thyroid peroxidase [216]. This cyanide-insensitive system appears to be located on the apical plasma membranes of thyroid cells and is NADPH-dependent ($K_m = 35$ μM) and Ca^{2+}-dependent, is stimulated by ATP and is inhibited by low

concentrations of pCMB [217–219]. Unlike the phagocyte oxidase, the thyroid system appears to possess intrinsic diaphorase activity and is not regulated by phorbol esters, but more probably by Ca^{2+}. Although H_2O_2 was originally described as being the product of the system, recent evidence using diacetyldeuterohaem-substituted horse-radish peroxidase as a trap for H_2O_2 and O_2^- suggests that the primary product is O_2^- [220] which then undergoes dismutation to provide the substrate for thyroid hormone synthesis. The more familiar assay for O_2^-, using the rate of superoxide dismutase (SOD)-sensitive cytochrome *c* reduction, had failed to detect O_2^- possibly due to high endogenous levels of SOD [219,221].

VI-B.2. Carotid body

The carotid body is able to recognize changes in oxygen tension in the arterial blood and produce nerve signals in order to regulate respiration and circulation. Absorbance spectra of perfused hypoxic carotid body preparations show spectral changes characteristic of flavin and haem which are suppressed by DPI, the inhibitor of the neutrophil O_2^- generating system [222], leading to the proposal that these systems might be related and that this carotid system is the chemoreceptor for p_{O_2}. It will be of interest to examine the system for evidence of O_2^- production (H_2O_2 has been detected by dihydrorhodamine oxidation), and to investigate the properties of the haem and flavin elements for comparison with the phagocytic oxidase components.

VI-B.3. Platelets

Like neutrophils, platelets exhibit a 'respiratory burst' of oxygen uptake after stimulation, but this oxygen is incorporated into arachidonic acid in order to synthesize products of the lipoxygenase and cyclo-oxygenase pathways. Although small amounts of O_2^- have been detected in platelet preparations it appears to be a byproduct of metabolism (possibly arising from mitochondrial or arachidonate metabolism) [223]. An inducible NADH-dependent, cyanide-insensitive H_2O_2 generating system has been reported in latex- or zymosan-stimulated platelets [224] but having a capacity equivalent to only 1% of the capacity of a neutrophil, its significance is not clear.

VI-B.4. Tumour cells

The observation that respiration of Ehrlich ascites tumour cells was not completely inhibited by mitochondrial inhibitors [225] implied that other electron transport systems are operating in these cells. The electron transport did not appear to be associated with cytochrome *P*-450 or cytochrome b_5 reductase, as only small amounts of these enzymes were detectable, and the respiratory system was not induced by phenobarbital as is cytochrome *P*-450 reductase. It was found that the isolated tumour cell nuclei respired with NADH or NADPH as substrates in a cyanide- and antimycin-resistant manner, and contained an autoxidizable *b*-type cytochrome with absorbance maxima at 559, 531 and 428 nm [226], very similar to the neutrophil cytochrome *b*-245 [21,23]. The NADPH-dependent activity was found to result in the production of O_2^- [227]. Another O_2^- generating activity in hepatoma nuclear membranes appears different from the Ehrlich cell system and may be associated with a microsomal fatty acid desaturase activity [228].

VII. Mammalian plasma membranes producing reduced oxygen species

VII-A. NADH-dependent

NADH-dependent oxidase activity has been demonstrated on mouse liver plasma membrane and clearly shown to be separate from microsomal or mitochondrial activity on the basis of subcellular fractionation studies and inhibitor profiles [229]. The activity was more resistant to pCMB and more susceptible to azide than the microsomal or mitochondrial activities and displayed a high diaphorase activity with ferricyanide as the acceptor. The natural acceptor was proposed to be oxygen. Although there is little flavin in mouse liver plasma membranes [230] the activity was inhibited by the flavoprotein inhibitor quinacrine (atebrin). Unlike the NADPH-dependent activity in adipocyte plasma membranes (see below), the NADH oxidase was either inhibited or stimulated by insulin depending on concentration. The presence of these hormone-sensitive redox systems has led to the proposal that they may be involved in signal transduction. Other suggested activities for these systems, which occur in a variety of tissues [231,232], are for the transport of cations by active [233] or passive mechanisms [234,235], or transport of amino acids [236,237]. Similar systems have been found in plant plasma membranes (see below).

VII-B. NADPH-dependent

A cyanide-resistant, NADPH-dependent oxidase activity has been described in plasma membranes of rat adipocytes. The activity was stimulated by insulin and by agents which mimic insulin action, but not by membrane perturbants. The measured product was H_2O_2 but it was uncertain if this was the primary product or whether it was derived from the dismutation of O_2^-. The activity of the oxidase system correlated with the inhibition of adenylate cyclase, possibly by a reversible oxidation of sulphydryl groups [238].

VII-C. NAD(P)H-dependent

Cytochemical staining with cerium chloride has demonstrated the presence of an O_2^- and H_2O_2-generating enzyme on the apical plasma membranes of absorptive cells of the rat small intestine [239]. The activity was sensitive to azide but not cyanide (at 10 mM) and could be distinguished from NADPH cytochrome *P*-450 reductase by its cellular location. The authors suggested that it may be closely related to the plasma membrane NADH oxidase activities described above.

VII-D. Other

Cultured arterial smooth muscle cells release O_2^- into their growth medium at a rate of 0.3–3 nmol O_2^-/min per mg cell protein [240]. This activity is largely dependent on the presence of cystine in the growth medium and is thought to result from the enzymic reduction of cystine to thiol compounds, possibly cysteine, with free S-H groups [241]. These can then undergo autoxidation to form O_2^- and regenerate dithiols [242,243].

VIII. Superoxide production by mitochondria and chloroplasts

In green plants the chloroplasts contain the machinery for the conversion of light energy into ATP while splitting water into oxygen, protons and electrons, the latter being used to drive the reduction of $NADP^+$ to NADPH. As oxygen is formed in the chloroplast its concentration is somewhat higher than that in the atmosphere, and this tends to promote 'leakage' of electrons from the components of the electron transport chain involved in the production of ATP, resulting in the formation of O_2^- [244,245]. One of these, ferredoxin, an iron-sulphur protein, has been shown to undergo autoxidation to produce O_2^- [11]. Little, if any of the O_2^- normally escapes from the chloroplast due to the presence of SOD and the impermeability of the chloroplast envelope, and in whole chloroplasts only H_2O_2 can be detected outside. This is generated entirely from O_2^- [246]. When plants are subjected to stress through drought, there is increased O_2^- production in chloroplasts, with a resultant increase in lipid peroxidation and cellular damage to which the plants respond by increasing their levels of antioxidant defence mechanisms [247,248].

The mitochondrial respiratory chain was first shown to produce reactive oxygen species by Loschen in 1971 [249]. Although the percentage of electrons which leak from the normal pathway is quite low (1–2%) [250], because of the high overall activity of mitochondrial metabolism, mitochondria produce considerable amounts of O_2^-, particularly when the electron flow is inhibited by agents such as antimycin, which cause the accumulation of reduced redox intermediates. There are two separate sites of O_2^- production; the flavoprotein, NADH dehydrogenase, and the ubiquinone-cytochrome *b* segment [251,252]. The former contributes about one-third of the O_2^- production and the latter two-thirds at physiological pH. It is not clear whether the source of O_2^- from the second site is from the autoxidation of ubisemiquinone or by the autoxidation of cytochrome *b*-566. Nohl and Jordan [253] have presented evidence that cytochrome *b*-566 is the more probable source and the mechanism is probably similar to that of cytochrome b_5 autoxidation. Little of the O_2^- formed in this way escapes the mitochondria due to the high levels of SOD which are maintained within the matrix. A role for O_2^- generation by mitochondria in the cytotoxicity of tumour necrosis factor (TNF) has been put forward by Wong [254]. TNF induces mitochondrial manganese-SOD which protects cells against subsequent susceptibility to TNF cytotoxicity. Antioxidants or anaerobiosis also block TNF cytotoxicity [255–257], as does pretreatment of cells with interleukin-1, which also induces manganese-SOD [258,259]. TNF increases radical production by susceptible cells, but the mechanism has not been elucidated [257].

Nohl has also reported another separate source of O_2^- associated with the outer membrane of rat heart mitochondria which is apparently quite distinct from the respiratory chain, since it was not inhibited by ethoxyformic anhydride [260]. This system is NADH-dependent and has a K_m for O_2 of 526 μM with a V_{max} of 8.33 nmol min per mg protein compared to the respiratory chain derived O_2^- of 7.69 nmol min per mg with a K_m of 645 μM (under 'classical conditions' in the presence of antimycin) and therefore represents a larger source of O_2^- in this tissue. It was not detected in rat liver mitochondria.

IX. Microsomal O_2^-; cytochrome *P*-450, cytochrome *P*-450 reductase and cytochrome b_5

The principal redox system of microsomes is that of the cytochrome *P*-450 family. These enzymes are responsible for both the detoxification of drugs and other xenobiotics, which is achieved with very broad specificity, and for the catalysis of oxidations of fatty acids and steroids which is achieved with very high specificity. There are now thought to be at least 60, and possibly more than 200, different cytochromes *P*-450 per mammalian species grouped into a number of gene families [261]. Cytochrome *P*-450 derives its name from the characteristic absorption band centred around 450 nm of the ferrous haem-carbon monoxide complex. The unusual spectral properties of the enzyme derive in part from a thiolate haem ligand [262]. Two discrete electron transfer reactions to cytochrome *P*-450 are required for the turnover of the enzyme. The usual source of the first

electron is NADPH via NADPH cytochrome *P*-450 reductase (also referred to as NADPH–cytochrome *c* reductase) with the second electron arising from either NADPH as above, or from NADH via cytochrome b_5 reductase and cytochrome b_5. All of these redox proteins are capable of O_2^- generation. Superoxide formation from liver microsomes is increased 8-fold in the livers from phenobarbitone-treated rats, due to the induction of cytochrome *P*-450 [263]. The rate of O_2^- from such microsomes is reported to be 9.6 nmol min per mg protein. Uncoupling of the monoxygenase system with drugs increases the rate of radical formation [264]. O_2^- can be produced both by dissociation of the oxygenated complex of reduced cytochrome *P*-450 [265] and from the autoxidation of cytochrome *P*-450 reductase [266–268] which contains both FAD and FMN. As is the case with other flavoproteins, the O_2^- is generated by the reaction of O_2 with flavin semiquinone.

Unlike the other haem proteins which have been shown to produce O_2^-, cytochrome b_5 has both the 5th and 6th ligand positions occupied by His-imidazole N atoms. In addition to its involvement in donation of the second electron to cytochrome *P*-450, cytochrome b_5 is involved in reduction of metHb in erythrocytes [269] and transfer of electrons to the cyanide-sensitive factor involved in fatty acid desaturase reactions [270]. Cytochrome b_5 is slowly autoxidizable [271,272] and the product has been shown to be O_2^- [273]. The rate of autoxidation is dependent on pH and ionic strength and increases with oxygen concentration, unlike that of Hb and Mb [274]. Under physiological conditions the rate of autoxidation is $5.6 \cdot 10^{-3}$ s^{-1}, about 60-fold faster than the rate of oxyferromyoglobin autoxidation. The high K_m for oxygen (230 μM) suggests the mechanism for O_2^- formation is by an outer sphere mechanism through the exposed haem edge [275], the same mechanism of electron transfer as is thought to be involved in the reduction of cytochrome *c* by O_2^- [276], although it has been argued that an inner sphere mechanism could be involved [277]. The redox potential of cytochrome b_5 is around 20 mV [278] and thus the reverse reaction (reduction of cytochrome b_5 by O_2^-) is favoured unless O_2^- is removed, this explains the increased rate of cytochrome b_5 autoxidation in the presence of adrenalin which scavenges O_2^- [273].

X. Production of superoxide by enzymes

X-A. Xanthine oxidase

A well-known biological source of O_2^- is xanthine oxidase (XO, eqn. 4), which is frequently used as a source of oxyradicals by researchers investigating the biological effects of O_2^-.

$$\text{xanthine} + H_2O + O_2 \xrightarrow{XO} \text{urate} + O_2^- \quad (4)$$

Unfortunately, it is often not appreciated that the major direct product of the reaction is H_2O_2, *not* O_2^- [279] and additionally the highly reactive $OH^{\cdot}$ can be formed [280]. The role of oxygen radical production from XO in reperfusion injury has received much attention recently (reviewed in Ref. 281). It has been shown that XO is formed from xanthine dehydrogenase (XDH) by proteolytic cleavage [282] or reversible oxidation of a reactive thiol [283–285] during tissue hypoxia and reperfusion with oxygenated blood. XDH normally oxidizes xanthine and other purines at the expense of NADH (Eqn. 5), and does not react with oxygen at a significant rate.

$$\text{xanthine} + NAD^+ + H_2O \xrightarrow{XDH} \text{urate} + NADH \quad (5)$$

This conversion can also apparently take place in endothelial cells in response to cytokines [194] and may contribute to their production of O_2^- (see above). The source of O_2^- in XO correlates with the amount of neutral flavin semiquinone ($FADH^{\cdot}$), whilst the H_2O_2 seems to be formed from a direct two-electron reduction of O_2 from fully reduced flavin [286]. XDH and XO are complex enzymes containing molybdenum and Fe/S redox centres in addition to the flavin centre, but these are not thought to directly interact with oxygen. No H_2O_2 or O_2^- was detected in experiments using the deflavo form of the enzyme [280].

X-B. Haemoglobin and myoglobin

Solutions of oxyhaemoglobin or oxymyoglobin yield O_2^- [287–289] where the reduced haem provides the single electron for the reduction of oxygen to O_2^-, with the haem Fe^{2+} being converted to the Fe^{3+} form (methaemoglobin, metmyoglobin). This process can be easily followed by spectrophotometry. Normally this reaction is slow in physiological situations but is substantially increased in the presence of certain anions such as cyanide or azide, or under acid conditions. In the normal red blood cell, the spontaneous formation of metHb is about 3% per day, but in the presence of cyanide at pH 5.0 the rate is increased 10^8-fold. Whilst anions have been observed binding to the met forms of the proteins, no evidence has been found for the binding of protons or anions to the reduced forms [290]. The mechanism of electron transfer from Fe^{2+} to O_2 is rather complex; although the Fe–O–O bond has a degree of Fe^{3+}–O_2^- character which would allow a simple dissociative mechanism, experimental evidence suggests direct involvement of a anion liganded to the haem iron as the electron donor to O_2 (reviewed by Caughey and Watkins [291]). To prevent the loss of oxygen-carrying capacity due to formation of metHb, the erythrocyte contains an enzyme, methaemoglobin reductase, to regenerate Hb.

XI. Superoxide production in plants

XI-A. NADH-dependent

Corn root protoplasts exhibit a NADH-dependent redox system similar to that seen in mammalian plasma membranes (above) which oxidizes external NADH at the expense of oxygen, and induces a hyperpolarization of the membrane [292] similar to that reported in diaphragm muscle membrane [235], probably as a result of increased K^+ uptake. The oxidation of NADH did not involve its transport into the protoplast and did not appear to be an artefact of protoplast isolation, since it also occurred in root segments, but it is difficult to rationalize how this system would be furnished with NADH in vivo. The identity of the reduced oxygen product has not been established. A seemingly identical system is present in carrot cells [293], where it was proposed that the oxidation of NADH was coupled to proton extrusion which is used to drive K^+ uptake. Recent reports implicate plasmalemma peroxidases as a source of some of these activities. They are able to oxidize NADH, yielding O_2^-; the latter can then dismute into H_2O_2 (and possibly participate in more complex radical chain reactions, see below) [294–299].

XI-B. NADPH-dependent

There are a number of NADPH-dependent O_2^--generating systems which have been described in plant plasma membranes.

XI-B.1. An inducible system in potato tuber tissues in response to infection

Potato tuber tissues exhibit a hypersensitive reaction in response to infection by incompatible races of fungi or hyphal wall components derived from them [300]. This hypersensitive reaction results in rapid cell death which is followed by resistance metabolism. The hypersensitive cell death was suppressed by SOD, thus suggesting O_2^- as a causal factor. Measurement of O_2^- production by protoplasts revealed an activity which was induced by hyphal wall components and was increased in the presence of NADPH [301]. It was insensitive to azide or other mitochondrial inhibitors, but was inhibited by thiol reagents. Compatible races of microorganisms possessed an inhibitory factor which prevented the development of the hypersensitivity reaction and decreased O_2^- production. The activity was also found in the leaves of potato, bean, pea, soyabean, tomato tobacco and sweet pepper plants and could be induced by digitonin, a membrane perturbing agent which stimulates the respiratory burst in phagocytes [302]. Digitonin pretreatment of leaves was found to prevent infection with fungi, the effect was reversed by SOD; therefore O_2^- production in plants appears to be a true defence mechanism.

XI-B.2. During lignification

Lignin biosynthesis provides an example of peroxidase-mediated O_2^- production which serves a useful purpose as opposed to the O_2^- production which occurs as a general side reaction in several peroxidases [303, 304], including enzymes of the cyclo-oxygenase and lipoxygenase pathways [305]. Lignification of plant cell walls involves the oxidative polymerization of phenols derived from cinnamic acid. It is initiated by the peroxidase-dependent formation of phenoxy radicals which requires H_2O_2 [306,307]. The H_2O_2 is generated by the oxidation of NADH produced by a bound malate dehydrogenase and is stimulated by monophenols and Mn^{2+} ions [295,308,309]. During the course of these reactions O_2^- is formed by the autoxidation of the NADH semiquinone ($NADH^{\cdot}$) and by the dissociation of O_2^- from peroxidase compound III, reactions that have been studied in detail by Halliwell and others [294,310]. Thus, oxygen radicals can be involved in both the synthesis and degradation of lignin (see below).

XI-B.3. A system involved in the uptake of ferric iron in roots

Iron-deficient plants can induce a system for the NADPH-dependent reduction of extracellular ferric chelates to ferrous ions which can then be taken up by the root cells [311–313]. Anaerobically the system reduces ferric chelates directly, but in the presence of oxygen at least part of the reduction process is mediated via O_2^- [314] and ferric reduction could be inhibited by superoxide dismutase. The contribution of O_2^- to the ferric reduction process was higher at lower ferric chelate concentrations and as the pH was increased from 5.5 to 7.1. Hendry has proposed that in waterlogged or wetland acid soils the increased Fe^{2+} uptake by intolerant plants can lead to a lethal level of oxygen-derived free radicals in plants not adapted to cope with these conditions, as the oxidation of ferrous ions in solution leads to O_2^- production ($Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^-$), and hydroxyl radical production ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}$) [315].

XII. The sea urchin respiratory burst

The earliest observations of cellular activation date back to Warburg's description of the "respiratory burst of fertilization" of sea-urchin eggs [316], which is now known to occur in the eggs of different species [317]. The purpose of the burst is to provide H_2O_2 for a peroxidase-mediated cross-linking of tyrosine residues of the glycoprotein coat of the egg surface to convert it to a rigid, relatively impermeable fertilization membrane [318]. Like the thyroid system, it is activated by

Ca^{2+} and ATP [319,320], is NADPH-dependent (K_m = 40 μM) and cyanide-insensitive, but unlike the thyroid it is inhibited by protein kinase C inhibitors. In fact, the susceptibility to these inhibitors and to phenothiazines closely parallels that of the phagocytic oxidase [320]. Although most authors suggest that O_2^- is not an intermediate in H_2O_2 production, a recent report suggests that O_2^- is present during the respiratory burst, although this may be derived from a reaction between ovoperoxidase and H_2O_2 [321]. In any case, the similarities between the NADPH-oxidase activities of the egg fertilization, the thyroid gland and the phagocyte O_2^- generating system deserve further investigation.

XIII. Formation of reduced oxygen species by microorganism

XII-A. Acanthamoeba

Amoebae share some striking behavioural similarities with neutrophils in that, during growth on bacteria, they must phagocytose, kill and digest their prey. Brooks and Schneider [322] have examined the biochemical parallels between the neutrophil and *Acanthamoeba castellanii* and have found an increase in respiration during phagocytosis of latex beads which, like the neutrophil oxidase, is insensitive to cyanide but sensitive to salicylhydroxamate and quercetin. The respiratory activity was concentrated in the phagolysozomal fraction of the disrupted cells, used NADPH as substrate, and was labile. Interestingly, the membrane fractions also contained a *b*-type cytochrome. Unfortunately, the authors do not report whether the product of the oxidase activity was O_2^-. Unlike the neutrophil, the unstimulated amoebae demonstrated a rather large cyanide-insensitive respiration.

XIII-B. White rot fungi

The white-rot fungi are capable of degrading both the polysaccharides (cellulose) and lignin present in wood. These extracellular enzyme systems are of great interest due to the large quantity of this valuable resource that is currently largely wasted due to the difficulty in extracting glucose feedstocks and other useful chemicals from it. This difficulty is a consequence of the crystallinity of the cellulose and its intimate association with lignin. One of the enzymes involved in the biological degradative process is cellobiose oxidase, whose function is to oxidise the reducing ends of cellodextrin chains and cellobiose to the corresponding lactones. Cellobiose oxidase is a glycoprotein of molecular mass 93 kDa [323] or 74 kDa which contains 1 mole each of FAD and haem [324]. Eriksson showed that in the presence of oxygen the rate of cellulose breakdown is more than doubled [325]. This observation, coupled with those which showed that a hydroxyl-radical-generating system increased cellulose susceptibility to cellulases due to the loss of hydrogen bonds and decreased ordered structure, has led to the suggestion that cellobiose oxidase might generate O_2^-, which in a series of reactions with Fe^{2+} as a catalyst could generate $OH^{\cdot}$. Evidence has been presented that this is the case [324,326,327]. The reduction of oxygen is inhibited by cyanide and the iron ligand α-bipyridyl, but there is no spectral evidence for the formation of a haem ligand. The redox potential of the haem is +115 mV at pH 7.0 (Cross, unpublished data), substantially higher than that of the neutrophil cytochrome *b*-245 and thermodynamically unfavourable for the production of O_2^- (E_{m7} = −160 mV [30]). It may be that O_2^- is formed by the reaction of O_2 with the flavin semiquinone. Cellobiose oxidase can be classified as a flavoprotein oxidase, since it forms a flavin sulphite adduct and stabilizes a red anionic semiquinone [324] (and thus differs from the neutrophil oxidase flavoprotein), but differs from other flavoprotein oxidases since it forms O_2^- rather than H_2O_2. In this behaviour it resembles 'old yellow enzyme' (EC 1.6.99.1) which also stabilizes a red semiquinone and yields O_2^- [81].

XIV. Hypothesis

The generation of O_2^- in many cell types (frequently associated with a b-type cytochrome) suggests a function in these cells distinct form the known microbicidal system present in the professional phagocytes. One attractive possibility is that of a role in cellular signalling. It has been shown that O_2^- causes proliferation of fibroblasts and this may be a more widespread phenomenon than is now appreciated. Fibroblasts are stimulated to produce O_2^- by cytokines which are released during inflammation. Another inflammatory mediator, interleukin-1, causes the proliferation of endothelial cells and stimulates O_2^- from them [190]. Similarly, oxidants have been shown to stimulate T-cell proliferation and activation [328], whilst antioxidants suppress proliferation and IL-1 synthesis [329]. O_2^- has been suggested to signal a increase in intracellular pH in certain cultured cells and spermatozoa, leading to cellular activation [210,330]. A role for O_2^- in cellular signalling may be similar to that which has now been discovered for NO (EDRF) possibly by the ligation of these small ligands with the Fe of the haem of GMP cyclase causing its stimulation or inhibition. Indeed, endothelium-derived vascular contraction factor ($EDCF_2$) has been suggested to be O_2^- [199]. Prostaglandin synthetase requires the continuous presence of lipid hydroperoxides and H_2O_2 for activity; these can be provided by neutrophils [331] and therefore by other O_2^--producing cells. It is not known whether or not the cytochrome which is involved in the fibroblast activity is identical to cytochrome

b-245, but it is known that mRNA coding for the haem-bearing subunit of cytochrome *b*-245 is expressed in a number of non-myeloid cell-types where it could conceivably participate in these activities [68,207]. A further signalling role for oxygen radicals may be that of the modification of low density lipoprotein (LDL), which promotes binding of the LDL to macrophage membranes. In atherosclerosis this leads to the uncontrolled uptake of large amounts of cholesterol by these cells, and their conversion to foam cells [332]. Alteration of LDL can be achieved by incubation with certain cell types; endothelial cells, smooth muscle cells and macrophages [333–336], all of which can generate O_2^- (see above), and seems to be dependent on the oxidation of LDL lipids [337]. A role for oxidants and plasma membrane redox activities in the control of growth and development has been reviewed by Ramasarma, and Crane and co-workers [231,232].

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References

1 Baccanari, D.P. (1978) Arch. Biochem. Biophys. 191, 351–357.
2 Fridovich, I. and Handler, P. (1961) J. Biol. Chem. 236, 1836–1840.
3 Ballou, D., Palmer, G. and Massey, V. (1969) Biochem. Biophys. Res. Commun. 36, 898–904.
4 Misra, H.P. and Fridovich, I. (1972) J. Biol. Chem. 247, 6960–6962.
5 Fisher, D.B. and Kaufman, S. (1973) J. Biol. Chem. 248, 4300–4304.
6 Nishikimi, M. (1975) Arch. Biochem. Biophys. 166, 273–279.
7 Halliwell, B. and Gutteridge, M.C. (1989) Free Radicals in Biology and Medicine, 2nd Edn, Oxford University Press, Oxford.
8 Kido, T., Soda, K., Suzuki, T. and Asada, K. (1976) J. Biol. Chem. 251, 6994–7000.
9 Ulrich, V. and Schnabel, K.H. (1973) Arch. Biochem. Biophys. 159, 240–248.
10 Hamilton, G.A. and Libby, R.D. (1973) Biochem. Biophys. Res. Commun. 55, 333–340.
11 Misra, H.P. and Fridovich, I. (1971) J. Biol. Chem. 246, 6686–6890.
12 Hirata, F. and Hayaishi, O. (1975) J. Biol. Chem. 250, 5960–5966.
13 Baldridge, C.W. and Gerrard, R.W. (1933) Am. J. Physiol. 103, 235–236.
14 Sbarra, A.J. and Karnovsky, M.L. (1959) J. Biol. Chem. 234, 1355–1362.
15 Hurst, J.K. and Barrette, W.C. (1989) Crit. Rev. Biochem. Mol. Biol. 24, 271–328.
16 Bellavite, P. (1988) Free Rad. Biol. Med. 4, 225–261.
17 Segal, A.W. (1989) J. Clin. Invest. 83, 1785–1793.
18 Hattori, H. (1961) Nugoya J. Med. Sci. 23, 362–378.
19 Shinagawa, Y., Tanaka, C., Teraoka, A. and Shinagawa, Y. (1966) J. Biochem. 59, 622–624.
20 Segal, A.W., Jones, O.T.G., Webster, D. and Allison, A.C. (1978) Lancet ii, 446–449.
21 Segal, A.W. and Jones, O.T.G. (1978) Nature 276, 515–517.
22 Borregaard, N., Johansen, K.S., Taudorff, E. and Wandell, J.H. (1979) Lancet i, 949–951.
23 Segal, A.W. and Jones, O.T.G. (1979) Biochem. J. 182, 181–188.
24 Segal, A.W. and Jones, O.T.G. (1980) Biochem. Biophys. Res. Commun. 92, 710–715.
25 Segal, A.W. and Jones, O.T.G. (1979) Biochem. Biophys. Res. Commun. 88, 130–136.
26 Segal, A.W. and Jones, O.T.G. (1980) FEBS Lett. 110, 111–114.
27 Hamers, M.N., De Boer, M., Meerhof, L.J., Weening, R.S. and Roos, D. (1984) Nature 307, 553–555.
28 Curnutte, J.T. (1988) Haematol./Oncol. Clin. N. America 2, 241–251.
29 Cross, A.R., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1981) Biochem. J. 194, 599–606.
30 Wood, P.M. (1974) FEBS Lett. 44, 22–24.
31 Segal, A.W., Garcia, R., Goldstone, A.H., Cross, A.R. and Jones, O.T.G. (1981) Biochem. J. 196, 363–367.
32 Roberts, P.J., Cross, A.R., Jones, O.T.G. and Segal, A.W. (1982) J. Cell Biol. 95, 720–729.
33 Andrew, P.W., Robertson, A.K., Lowrie, D.B., Cross, A.R. and Jones, O.T.G. (1987) Biochem. J. 248, 281–283.
34 Cross, A.R., Higson, F.K., Jones, O.T.G., Harper, A.M. and Segal, A.W. (1982) Biochem. J. 204, 479–485.
35 Gabig, T.G., Schervish, E.W. and Santinga, J.T. (1982) J. Biol. Chem. 257, 4114–4119.
36 Bellavite, P., Cross, A.R., Serra, M.C., Davioli, P., Jones, O.T.G. and Rossi, F. (1983) Biochim. Biophys. Acta 746, 40–47.
37 Kuyotaki, C., Peisach, J. and Bloom, B.R. (1984) J. Immunol. 132, 857–866.
38 Serra, M.C., Bellavite, P., Davoli, A., Bannister, J.V. and Rossi, F. (1984) Biochim. Biophys. Acta 788, 138–146.
39 Lutter, R., van Schaik, M., van Zweiten, R., Wever, R., Roos, D. and Hamers, M.N. (1985) J. Biol. Chem. 260, 2237–2244.
40 Morel, F. and Vignais, P.V. (1984) Biochim. Biophys. Acta 764, 213–225.
41 Pember, S.O., Heyl, B.L., Kinkade, J.M. and Lambeth, J.D. (1984) J. Biol. Chem. 259, 10590–10595.
42 Morel, F., Doussière, J., Stasia, M.-J. and Vignais, P.V. (1985) Eur. J. Biochem. 152, 669–679.
43 Yamaguchi, T., Hayakawa, T., Kaneda, M., Kakinuma, K. and Yoshikawa, A. (1989) J. Biol. Chem. 264, 112–118.
44 Gabig, T.G., Bearman, S.I. and Babior, B.M. (1979) Blood 53, 1133–1139.
45 Edwards, S.W., Hallett, M.B., Lloyd, D. and Campbell, A.K. (1983) FEBS Lett. 161, 60–64.
46 Kakinuma, K., Kaneda, M., Chiba, T. and Ohnishi, T. (1986) J. Biol. Chem. 261, 9426–9432.
47 Testa, B. and Jenner, P. (1981) Drug. Metab. Rev. 12, 1–117.
48 Collman, J.P., Brauman, J.I., Halbert, T.R. and Suslick, K.S. (1976) Proc. Natl. Acad. Sci. USA 73, 3333–3337.
49 Iizuka, T., Kanegasaki, S., Makino, R., Tanaica, T. and Ishimura, Y. (1985) Biochem. Biophys. Res. Commun. 130, 621–626.
50 Cross, A.R., Parkinson, J.F. and Jones, O.T.G. (1984) Biochem. J. 223, 337–344.
51 Light, D.R., Walsh, C., O'Callaghan, A.M., Goetzl, E.J. and Tauber, A.I. (1981) Biochemistry 20, 1468–1476.
52 Gabig, T.G. and Lefker, B.A. (1984) Biochem. Biophys. Res. Commun. 118, 430–436.
53 Cross, A.R., Parkinson, J.F. and Jones, O.T.G. (1985) Biochem. J. 226, 881–884.
54 Bellavite, P., Cassatella, M.A., Papini, E., Megyeri, P. and Rossi, F. (1986) FEBS Lett. 199, 159–163.
55 Bellavite, P., Jones, O.T.G., Cross, A.R., Papini, E. and Rossi, F. (1984) Biochem. J. 223, 639–648.
56 Berton, G., Papini, E., Cassatella, M.A., Bellavite, P. and Rossi, F. (1985) Biochim. Biophys. Acta 810, 164–173.

57 Green, T.R. and Pratt, K.L. (1988) J. Biol. Chem. 263, 5617–5623.
58 Gabig, T.G. and Lefker, B.A. (1985) J. Biol. Chem. 260, 3991–3995.
59 Wakeyama, H., Takeshige, K., Takanagi, R. and Minakami, S. (1982) Biochem. J. 205, 593–601.
60 Bellavite, P., Serra, M.C., Davioli, A., Bannister, J.V. and Rossi, F. (1983) Mol. Cell. Biochem. 52, 17–25.
61 Markert, M., Glass, G.A. and Babior, B.M. (1985) Proc. Natl. Acad. Sci. USA 82, 3144–3148.
62 Glass, G.A., De Lisle, D.M., De Togni, P., Gabig, T.G., Magee, B.E., Markert, M. and Babior, B.M. (1986) J. Biol. Chem. 261, 13247–13251.
63 Doussière, J. and Vignais, P.V. (1985) Biochemistry 24, 7231–7239.
64 Segal, A.W. (1987) Nature 326, 88–91.
65 Parkos, C.A., Allen, R.C., Cochrane, C.G. and Jesaitis, A.J. (1987) J. Clin. Invest. 80, 732–742.
66 Parkos, C.A., Allen, R.A., Cochrane, C.G. and Jesaitis, A.J. (1988) Biochim. Biophys. Acta 932, 71–83.
67 Royer-Pokora, B., Kunkel, L.M., Monaco, A.P., Goff, S.C., Newburger, P.E., Baehner, R.L., Cole, F.S., Curnutte, J.T. and Orkin, S.H. (1986) Nature 322, 32–38.
68 Parkos, C.A., Dinauer, M.C., Walker, L.E., Allen, R.A., Jesaitis, A.J. and Orkin, S.H. (1988) Proc. Natl. Acad. Sci. USA 85, 3319–3323.
69 Perin, M.S., Fried, V.A., Slaughter, C.A. and Sudhof, T.C. (1988) EMBO J. 7, 2697–2703.
70 Nugent, J.H.A., Gratzer, W. and Segal, A.W. (1989) Biochem. J. 264, 921–924.
71 Newburger, P.E., Ezekowitz, R.A.B., Whitney, C., Wright, J. and Orkin, S.H. (1988) Proc. Natl. Acad. Sci. (USA) 85, 5215–5219.
72 Babior, B.M. and Kipnes, R.S. (1977) Blood 50, 517–524.
73 Gabig, T.G. and Babior, B.M. (1979) J. Biol. Chem. 254, 9070–9074.
74 Parkinson, J.F. and Gabig, T.G. (1988) J. Biol. Chem. 263, 8859–8863.
75 Cross, A.R., Jones, O.T.G., Garcia, R. and Segal, A.W. (1982) Biochem. J. 208, 759–763.
76 Gabig, T.G. (1983) J. Biol. Chem. 258, 6352–6356.
77 Gabig, T.G. and Lefker, B.A. (1984) J. Clin. Invest. 73, 701–705.
78 Ohno, Y., Buescher, E.S., Roberts, R., Metcalf, J.A. and Gallin, J.I. (1986) Blood 67, 1132–1138.
79 Parkinson, J.F. and Gabig, T.G. (1988) J. Bioenerg. Biomemb. 20, 653–677.
80 Yea, C.M., Cross, A.R. and Jones, O.T.G. (1990) Biochem. J. 265, 95–100.
81 Massey, V. and Hemmerich, P. (1980) Biochem. Soc. Trans. 8, 246–257.
82 Fukuhara, Y., Ise, Y. and Kakinuma, K. (1988) FEBS Lett. 229, 150–156.
83 Sakane, F., Takahashi, K. and Koyama, J. (1984) J. Biochem. 96, 671–678.
84 Kakinuma, K., Fukuhara, Y. and Kaneda, M. (1987) J. Biol. Chem. 262, 12316–12322.
85 Takasugi, S., Ishida, K., Takeshige, K. and Minakami, S. (1989) J. Biochem. 105, 155–157.
86 Doussière, J. and Vignais, P.V. (1986) Biochem. Biophys. Res. Commun. 139, 85–93.
87 Umei, T., Takeshige, K. and Minakami, S. (1986) J. Biol. Chem. 261, 5229–5232.
88 Smith, R.M., Curnutte, J.T. and Babior, B.M. (1989) J. Biol. Chem. 264, 1958–1962.
89 Cross, A.R. and Jones, O.T.G. (1986) Biochem. J. 237, 111–116.
90 Hancock, J.T. and Jones, O.T.G. (1987) Biochem. J. 242, 103–107.
91 Cross, A.R., Yea, C.M., Jones, O.T.G. and Shute, J. (1989) Eur. J. Clin. Invest. 19, A61.
92 Millard, J.A., Gerard, K.W. and Schneider, D.L. (1979) Biochem. Biophys. Res. Commun. 80, 312–319.
93 Crawford, D.R. and Schneider, D.L. (1981) Biochem. Biophys. Res. Commun. 99, 1277–1286.
94 Sloan, E.P., Crawford, D.R. and Schneider, D.L. (1981) J. Exp. Med. 153, 1316–1328.
95 Crawford, D.R. and Schneider, D.L. (1982) J. Biol. Chem. 257, 6662–6668.
96 Cunningham, C.C., DeChatelet, L.R., Spach, P.I., Pane, J.W., Thomas, M.J., Lees, C.J. and Shirley, P.S. (1982) Biochim. Biophys. Acta 682, 430–435.
97 Cross, A.R., Jones, O.T.G., Garcia, R. and Segal, A.W. (1983) Biochem. J. 216, 765–768.
98 Lutter, R., van Zweiten, R., Weening, R.S., Hamers, M.N. and Roos, D. (1984) J. Biol. Chem. 259, 9603–9606.
99 Bromberg, Y. and Pick, E. (1984) Cellular Immunol. 88, 213–221.
100 Heyneman, R.A. and Vercauteren, R.E. (1984) J. Leukocyte Biol. 36, 751–759.
101 McPhail, L.C., Shirley, P.S., Clayton, C.C. and Snyderman, R. (1985) J. Clin. Invest. 75, 1735–1739.
102 Curnutte, J.T. (1985) J. Clin. Invest. 75, 1740–1743.
103 Curnutte, J.T., Kuver, R. and Scott, P.J. (1987) J. Biol. Chem. 262, 5563–5569.
104 Clark, R.A., Leidal, K.G., Pearson, D.W. and Nauseef, W.M. (1987) J. Biol. Chem. 262, 4065–4074.
105 Gabig, T.G., English, D., Akard, L.P. and Schell, M.J. (1987) J. Biol. Chem. 262, 1685–1690.
106 Babior, B.M., Kuver, R. and Curnutte, J.T. (1988) J. Biol. Chem. 263, 1713–1718.
107 Volpp, B.D., Nauseef, W.M. and Clark, R.A. (1988) Science 242, 1295–1297.
108 Nunoi, H., Rotrosen, D., Gallin, J.I. and Malech, H.L. (1988) Science 242, 1298–1301.
109 Curnutte, J.T., Berkow, R.L., Roberts, R.L., Shurin, S.B. and Scott, P.J. (1988) J. Clin. Invest. 81, 606–610.
110 Sechler, J.M.G., Malech, H.L., White, C.J. and Gallin, J.I. (1988) Proc. Natl. Acad. Sci. USA 85, 4874–4878.
111 Bolscher, B.G.J.M., van Zweiten, R., Kramer, I.M., Weening, R.S., Verhoeven, A.J. and Roos, D. (1989) J. Clin. Invest. 83, 757–763.
112 Leto, T.L., Lomax, K.J., Volpp, B.D., Nunoi, H., Sechler, J.M.G., Nauseef, W.M., Clark, R.A., Gallin, J.I. and Malech, H.L. (1990) Science 248, 727–730.
113 Quinn, M.T., Parkos, C.A., Walker, L., Orkin, S.H., Dinauer, M.C. and Jesaitis, A.J. (1989) Nature 342, 198–200.
114 Seifert, R., Rosenthal, W. and Schultz, G. (1986) FEBS Lett. 205, 161–165.
115 Seifert, R. and Schultz, G. (1987) Biochem. Biophys. Res. Commun. 146, 1296–1302.
116 Seifert, R. and Schultz, G. (1987) Eur. J. Biochem. 162, 563–569.
117 Doussière, J. and Vignais, P.V. (1988) FEBS Lett. 234, 362–366.
118 Ligeti, E., Doussière, J. and Vignais, P.V. (1988) Biochemistry 27, 192–200.
119 Ligeti, E., Tardif, M. and Vignais, P.V. (1989) Biochemistry 28, 7116–7123.
120 Ishida, K., Takeshige, K., Takasugi, S. and Minakami, S. (1989) FEBS Lett. 243, 169–172.
121 Curnutte, J.T., Scott, P.J. and Mayo, L.A. (1989) Proc. Natl. Acad. Sci. USA 86, 825–829.
122 Curnutte, J.T., Scott, P.J. and Babior, B.M. (1989) J. Clin. Invest. 83, 1236–1240.
123 Smith, R.M., Curnutte, J.T., Mayo, L.A. and Babior, B.M. (1989) J. Biol. Chem. 264, 12243–12248.
124 Koppenol, W.H. (1989) in CRC Critical Reviews in Membrane Lipid Oxidation, Vol. 1, pp. 1–13, CRC, Boca Raton.
125 Parkinson, J.F. (1985) Ph. D. Thesis, University of Bristol.

126 Cross, A.R. (1987) Biochem. Pharmacol. 36, 489–493.
127 Cross, A.R., Yea, C.M. and Jones, O.T.G. (1988) Biochem. Soc. Trans. 16, 888–889.
128 Korchak, H.M. and Weissmann, G. (1979) Proc. Natl. Acad. Sci. USA 75, 3818–3822.
129 Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1088–1096.
130 Korchak, H.M. and Weissmann, G. (1980) Biochim. Biophys. Acta 601, 180–196.
131 Naccache, P., Showell, H.J., Becker, E.L. and Sha'afi, R.I. (1977) J. Cell Biol. 75, 635–649.
132 Lazzari, K.G., Proto, P.J. and Simons, E.R. (1986) J. Biol. Chem. 261, 9710–9713.
133 Seligmann, B.E. and Gallin, J.I. (1980) J. Clin. Invest. 66, 493–503.
134 Tauber, A.I. and Simons, E.R. (1985) FEBS Lett. 156, 161–164.
135 Cameron, A.R., Nelson, J. and Forman, H.J. (1983) Proc. Natl. Acad. Sci. (USA) 80, 3726–3728.
135 Whitin, J.C., Chapman, C.E., Simons, E.R., Chovaniec, M.E. and Cohen, H.J. (1980) J. Biol. Chem. 255, 1874–1878.
136 Roos, D., Voetman, A.A. and Meerhof, L.J. (1983) J. Cell Biol. 97, 368–377.
137 Henderson, L.M., Chappell, J.B. and Jones, O.T.G. (1987) Biochem. J. 246, 325–329.
138 Henderson, L.M., Chappell, J.B. and Jones, O.T.G. (1988) Biochem. J. 255, 285–290.
139 Grinstein, S. and Furuya, W. (1986) Am. J. Physiol. 251, C55-C65.
140 Grinstein, S., Furuya, W. and Biggar, W.D. (1986) J. Biol. Chem. 261, 512–514.
141 Henderson, L.M., Chappell, J.B. and Jones, O.T.G. (1989) Biochem. J. 264, 249–255.
142 Cross, A.R. (1990) Free Radical Biol. Med. 8, 71–93.
143 Seifert, R. and Schachtele, C. (1988) Biochem. Biophys. Res. Commun. 152, 585–592.
144 Rossi, F. (1986) Biochem. Biophys. Acta 853, 65–89.
145 Becker, E.L. (1979) J. Reticuloendothel. Soc. 26, 701–709.
146 Vercellotti, G.M., Yin, H.Q., Gustafson, K.S., Nelson, R.D. and Jacobs, H.S. (1988) Blood 71, 1100–1107.
147 Minta, J.O. and Williams, M.D. (1986) J. Rheumatol, 13, 498–504.
148 Sung, C-P, Mirabelli, C.K. and Badger, A.M. (1984) J. Rheumatol. 11, 153–157.
149 Hafstrom, I., Seligmann, B.E., Friedman, M.M. and Gallin, J.I. (1984) J. Immunol. 132, 2007–2014.
150 Neal, T.M., Vissers, M.C.M. and Winterbourne, C.C. (1987) Biochem. Pharmacol. 36, 2511–2517.
151 Coates, T.D., Wolach, B., Tzeng, D.Y., Higgins, C., Baehner, R.L. and Boxer, L.A. (1987) Blood 62, 1070–1077.
152 Mirabelli, C.K., Sung, C-P., Picker, D.H., Barnard, C., Hydes, P. and Badger, A.M. (1988) J. Rheumatol. 15, 1064–1069.
153 Oyanagui, Y. (1978) Biochem. Pharmacol. 27, 777–782.
154 Perianin, A., Gougerot-Pocidalo, M-A. and Hakim, J. (1987) Biochem. Pharmacol. 36, 2609–2615.
155 Davis, P., Johnston, C., Miller, C.L. and Wong, K. (1983) Arthritis Rheum. 26, 82–86.
156 Blackburn, W.D. and Heck, L.W. (1988) Biochem. Biophys. Res. Commun. 152, 136–142.
157 Molski, R.F.P., Naccache, P.H., Marsh, M.L., Kermode, J., Becker, E.L. and Sha'afi, R.I. (1984) Biochem. Biophys. Res. Commun. 124, 644–650.
158 Holian, A. (1986) FEBS Lett. 201, 15–19.
159 Tsunawaki, S. and Nathan, C.F. (1986) J. Biol. Chem. 261, 11536–11570.
160 Maridonneau-Parini, I. and Tauber, A.I. (1986) Biochem. Biophys. Res. Commun. 138, 1099–1105.
161 Smolen, J.E. and Weissmann, G. (1980) Biochem. Pharmacol. 29, 533–538.
162 Della Bianca, V., Grzeskowiak, M., DeTogni, P., Cassatella, M. and Rossi, F. (1985) Biochem. Biophys. Acta 845, 223–236.
163 Otha, H., Okajima, F. and Ui, M. (1985) J. Biol. Chem. 260, 15771–15780.
164 Wright, C.D., Hoffman, M.D., Theuson, D.O. and Conroy, M.C. (1987) J. Leuk. Biol. 42, 30–35.
165 Wright, C.D., Hoffman, M.D., Theuson, D.O. and Conroy, M.C. (1987) Biochem. Biophys. Res. Commun. 148, 1110–1117.
166 Gerard, C., McPhail, L.C., Marfat, A., Stimler-Gerard, N.D., Bass, D.A. and McCall, C.E. (1986) J. Clin. Invest. 77, 61–65.
167 Maridonneau-Parini, I., Tringale, S.M. and Tauber, A.I. (1986) J. Immunol. 137, 2925–2929.
168 Cooke, E. and Hallett, M.B. (1985) Biochem. J. 232, 323–327.
169 Wilson, E., Olcott, M.C., Bell, R.M., Merrill, A.J. and Lambeth, J.D. (1986) J. Biol. Chem. 261, 12616–2623.
170 Wright, C.D. and Hoffman, M.D. (1986) Biochem. Biophys. Res. Commun. 135, 749–755.
171 Aviram, I., Simons, E.R. and Babior, B.M. (1984) J. Biol. Chem. 259, 306–311.
172 Simchowitz, L., Mehta, J. and Spilberg, I. (1979) Arthritis Rheum. 22, 755–763.
173 Rest, R.F., Farrell, C.F. and Naids, F.L. (1988) J. Leuk. Biol. 43, 158–164.
174 Nakagawara, A. and Minikamai, S. (1975) Biochem. Biophys. Res. Commun. 64, 760–767.
175 Yamaguchi, T. and Kakinuma, K. (1982) Biochem. Biophys. Res. Commun. 104, 200–205.
176 Wakeyama, H., Takeshige, K. and Minakami, S. (1983) Biochem J. 210, 577–581.
177 Green, T.R., Wu, D.E. and Wirtz, M.K. (1983) Biochem. Biophys. Res. Commun. 110, 973–978.
178 Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1088–1096.
179 Cotgreave, I.A., Duddy, S.K., Kass, G.E.N., Thompson, D. and Moldeus, P. (1989) Biochem. Pharmacol. 38, 649–656.
180 Volkman, D.J., Buescher, E.S., Gallin, J.I. and Fauci, A.S. (1984) J. Immunol. 133, 3006–3009.
181 Maly, F-E., Cross, A.R., Jones, O.T.G., Wolf-Vorbeck, G., Walker, C., Dahinden, C.A. and De Weck, A.L. (1988) J. Immunol. 140, 2334–2339.
182 Hancock, J.T., Maly, F-E. and Jones, O.T.G. (1989) Biochem. J. 262, 373–375.
183 Maly, F-E., Jones, O.T.G., Gauchat, J-F., Cross, A.R., Urwyler, A., Walker, C., Dahinden, C.A., De Weck, A.L. and Nakamura, M. (1989) J. Immunol. 142, 1260–1267.
184 Hancock, J.T., Henderson, L.M. and Jones, O.T.G. (1990) Immunology 71, 213–217.
185 Kobayashi, S., Imajoh-Ohmi, S., Nakamura, M. and Kanegasaki, S. (1990) Blood 75, 458–461.
186 Murrell, G.A.C., Francis, M.J.O. and Bromley, L. (1989) Biochem. Soc. Trans. 17, 483.
187 Meier, B., Radecke, H.H., Selle, S., Younes, M., Sies, H., Resch, K. and Habermehl, G.G. (1989) Biochem. J. 263, 539–545.
188 Klebanoff, S.J., Vadas, M.A., Harlan, J.M., Sparks, L.H., Gamble, J.R., Agosti, J.M. and Waltersdorph, A.M. (1986) J. Immunol. 136, 4220–4225.
189 Tsujimoto, M., Yokota, S., Vilcek, J. and Weissmann, G. (1986) Biochem. Biophys. Res. Commun. 137, 1094–1100.
190 Matsubara, T. and Ziff, M. (1986) J. Immunol. 137, 3295–3298.
191 Nathan, C.F. (1989) Blood 73, 301–306.
192 Murrell, G.A.C., Francis, M.J.O. and Bromley, L. (1989) Biochem. Soc. Trans. 17, 484.
193 Ooi, B.S., MacCarthy, E.P., Hsu, A. and Ooi, Y.M. (1983) J. Lab. Clin. Med. 102, 428–433.
194 Friedl, H.P., Till, G.O., Ryan, U.S. and Ward, P.A. (1989) FASEB J. 3, 2512–2518.

195 Kontos, H.A. (1989) Chem. Biol. Interact. 72, 229–255.
196 Perez, H.D., Weksler, B.B. and Goldstein, I.A. (1980) Inflammation 4, 313–328.
197 Petrone, W.F., English, D.K., Wong, K., Wong, K. and McCord, J.M. (1980) Proc. Natl. Acad. Sci. USA 77, 1159–1163.
198 Phan, S.H., Gannon, D.E., Varani, J., Ryan, U.S. and Ward, P.A. (1989) Am. J. Pathol. 134, 1201–1211.
199 Vanhoutte, P.M. and Katusic, Z.S. (1988) Trends Pharm. Sci. 9, 229–230.
200 Saran, M. and Bors, W. (1989) Free Radical Biol. Med. 7, 213–220.
201 Shah, S.V. (1981) J. Lab. Clin. Med. 98, 46–57.
202 Basci, A. and Shah, S.V. (1987) Am. J. Physiol. 252, C611–C617.
203 Adler, S., Baker, P.J., Johnson R.J., Ochi, R.F., Pritzl, P. and Couser, W.G. (1986) J. Clin. Invest. 77, 762–767.
204 Baud, L., Perez, J. and Ardaillou, R. (1986) Am. J. Physiol. 250, F596–F604.
205 Sedor, J.R. and Abboud, H.E. (1984) Kidney Intl. 25, 218 (Abst.)
206 Miyanoshita, A., Takahashi, T. and Minakami, H. (1989) Biochem. Biophys. Res. Commun. 165, 519–525.
207 Sumimoto, H., Nozaki, M., Sasaki, H., Takeshige, K., Sakaki, Y. and Minakami, S. (1989) Biochem. Biophys. Res. Commun. 165, 902–906.
208 Tosic, J. and Walton, A. (1950) Biochem. J. 47, 199–212.
209 Holland, M.K. and Storey, B.T. (1981) Biochem. J. 198, 273–280.
210 Aitken, R.J. and Clarkson, J.S. (1987) J. Reprod. Fert. 81, 459–469.
211 Kumar, P.G., Laloraya, M. and Laloraya, M.M. (1989) Biochem. Biophys. Res. Commun. 161, 771–775.
212 Sandhu, S.S., Band, A., Abayasekera, D.R.E., Rice-Evans, C.E. and Cooke, B.A. (1989) Biochem. Soc. Trans. 17, 710–711.
213 Smith, W.L. and Lands, W.E.M. (1972) J. Biol. Chem. 247, 1038–1047.
214 Lands, W.E.M. (1985) Free Radicals Biol. Med. 1, 97–101.
215 Laloraya, M., Kumar, G.P. and Laloraya, M. (1989) Biochem. Biophys. Acta 161, 762–770.
216 Taurog, A. (1970) Recent Prog. Horm. Res. 26, 189–247.
217 Dème, D., Virion, A., Ait Hammou, N. and Pommier, J. (1985) FEBS Lett. 186, 107–110.
218 Dupuy, C., Virion, A., Hammou, N.A., Kaniewski, J., Dème, D. and Pommier, J. (1986) Biochem. Biophys. Res. Commun. 141, 839–846.
219 Nakamura, Y., Ogihara, S. and Ohtaki, S. (1987) J. Biochem. 102, 1121–1132.
220 Nakamura, Y., Ohtaki, S., Makino, R., Tanaka, T. and Ishimura, Y. (1989) J. Biol. Chem. 264, 4759–4761.
221 Dupuy, C., Kaniewski, J., Dème, D., Pommier, J. and Virion, A. (1989) Eur. J. Biochem. 185, 597–603.
222 Acker, H., Dufau, E., Huber, J. and Sylvester, D. (1989) FEBS Lett. 256, 75–78.
223 Marcus, A.J. (1979) Seminars Hematol. 16, 188–195.
224 Finazzi-Agrò, A., Menichelli, A., Persiani, M., Biancini, G. and Del Principe, D. (1982) Biochim. Biophys. Acta 718, 21–25.
225 Borst, P. (1962) Biochim. Biophys. Acta 57, 270–282.
226 Bartoli, G.M., Dani, A., Galeotti, T., Russo, M. and Terranova, T. (1975) Z. Krebsforsch. 83, 223–231.
227 Bartoli, G.M., Galeotti, T. and Azzi, A. (1977) Biochim. Biophys. Acta 497, 622–626.
228 Peskin, A.V., Zoarsky, I.B. and Konstantinou, A. (1980) FEBS Lett. 117, 44–48.
229 Goldenberg, H., Crane, F.L. and Morré, D.J. (1979) J. Biol. Chem. 254, 2491–2498.
230 Jarasch, E-D., Kartenbeck, J., Bruder, G., Fink, A., Morré, D.J. and Franke, W.W. (1979) J. Cell Biol. 80, 37–52.
231 Crane, F.L., Sun, I.L., Clark, M.G., Grebing, C. and Löw, H. (1985) Biochim. Biophys. Acta 811, 233–264.
232 Ramasarma, T. (1982) Biochim. Biophys. Acta 694, 69–93.
233 Skou, J.C., (1965) Physiol. Rev. 45, 596–617.
234 Garcia-Sancho, J., Sanchez, A. and Herreros, B. (1979) Biochim. Biophys. Acta556, 118–130.
235 Zemkova, H., Teisinger, J. and Vyskocil, F. (1984) Biochim. Biophys. Acta 775, 64–70.
236 Garcia-Sancho, J., Sanchez, A., Handlogten, M.E. and Christensen, H.N. (1977) Proc. Natl. Acad. Sci. USA 74, 1488–1491.
237 Yamamoto, S-I. and Kawaski, T. (1981) Biochim. Biophys. Acta 644, 192–200.
238 Mukherjee, S.P. and Lynn, W.S. (1977) Arch. Biochem. Biophys. 184, 69–76.
239 Ishikawa, Y., Hirai, K-I. and Ogawa, K. (1981) Acta Histochem. Cytochem. 14, 727–733.
240 Heinecke, J.W., Baker, L. Rosen, H. and Chait, A. (1986) J. Clin. Invest. 77, 757–761.
241 Heinecke, J.W., Rosen, H., Suzuki, L.A. and Chait, A. (1987) J. Biol. Chem. 262, 10098–10103.
242 Misra, H.P. (1974) J. Biol. Chem. 249, 2151–2155.
243 Rowley, D.A. and Halliwell, B. (1982) FEBS Lett. 138, 33–36.
244 Asada, K. and Kiso, K. (1973) Agric. Biol. Chem. 37, 453–459.
245 Elstner, E.F. (1982) Annu. Rev. Plant Physiol. 33, 73–96.
246 Asada, K., Kiso, K. and Yoshikawa, K. (1974) J. Biol. Chem. 249, 2175–2181.
247 Price, A.H. and Hendry, G.A.F. (1989) Biochem. Soc. Trans. 17, 493–494.
248 Price, A.H., Atherton, N.M. and Hendry, G.A.F. (1989) Free Radical Res. Commun. 8, 61–66.
249 Loschen, G., Flohe, L. and Chance, B. (1971) FEBS Lett. 18, 261–264.
250 Boveris, A., and Chance, B. (1973) Biochem. J. 134, 707–716.
251 Boveris, A., Cadenas, E. and Stoppani, A.O.M. (1976) Biochem. J. 156 435–444.
252 Turrens, J.F. and Boveris, A. (1980) Biochem. J. 191, 421–427.
253 Nohl, H. and Jordan, W. (1986) Biochem. Biophys. Res. Commun. 138, 533–539.
254 Wong, G.H.W., Elwell, J.H., Oberley, L.W. and Goeddel, D.V. (1989) Cell 58, 923–931.
255 Matthews, N., Neale, M.L., Jackson, S.K. and Stark, J.M. (1982) Immunology 62, 153–155.
256 Satomi, N., Sakurai, A., Haranaka, R. and Haranaka, K. (1988) J. Biol. Response Mod. 7, 54–63.
257 Yamauchi, N., Kuriyama, H., Watanabe, N., Neda, H., Maeda, M. and Nitsui, Y. (1989) Cancer Res. 43, 1671–1675.
258 Wong, G.H.W. and Goeddel, D.V. (1988) Science 242, 941–944.
259 Masuda, A., Longo, D.L., Kobayashi, Y., Apella, E., Oppenheim, J.J. and Matsusima, K. (1988) FASEB J. 2, 3087–3091.
260 Nohl, H. (1987) FEBS Lett. 214, 269–273.
261 Nebert, D.W., Nelson, D.R. and Feyereisen, R. (1989) Xenobiotica 19, 1149–1160.
262 Dawson, J.H. and Eble, K.S. (1986) Adv. Inorg. Bioinorg. Mech. 4, 1–64.
263 Auclair, C., DeProst, D. and Hakim, J. (1978) Biochem. Pharmacol. 27, 355–358.
264 Kuthan, H., Ullrich, V. and Estabrook, R.W. (1982) Biochem. J. 203, 551–558.
265 Sligar, S.G., Lipscomb, J.D., Debrunner, P.G. and Gunsalus, I.C. (1974) Biochem. Biophys. Res. Commun. 61, 290–296.
266 Nakamura, S. and Yamazaki, I. (1969) Biochim. Biophys. Acta 189, 29–37.
267 Aust, S.D., Roerig, D.L. and Pederson, T.C. (1972) Biochem. Biophys. Res. Commun. 47, 1133–1137.
268 Debey, P. and Balny, C. (1973) Biochimie 55, 329–332.
269 Hultquist, D.E. and Passon, P.G. (1971) Nature 229, 252–254.
270 Oshino, N., Imai, Y. and Sato, R. (1971) J. Biochem. 69, 155–167.
271 Boveris, A., Oshino, N. and Chance, B. (1972) Biochem. J. 128, 617–630.

272 Lemberg, R. and Barratt, J. (1973) Cytochromes, Academic Press, London.
273 Berman, M.C., Adnams, C.M., Ivanetich, K.M. and Kench, J.E. (1976) Biochem. J. 157, 237–246.
274 George, P. and Stratman, C.J. (1952) Biochem. J. 51, 418–425.
275 Bennett, L.E. (1973) Prog. Inorg. Chem. 18, 1–176.
276 Butler, J., Koppenol, W.H. and Margoliash, E. (1982) J. Biol. Chem. 257, 10747–10750.
277 Bartnicki, E.W., Belser, N.O. and Castro, C.E. (1978) Biochemistry 17, 5582–5586.
278 Velick, S.F. and Strittmatter, P. (1956) J. Biol. Chem. 221, 265–275.
279 Fridovich, I. (1970) J. Biol. Chem. 245, 4053–4057.
280 Kuppusamy, P. and Zweier, J.L. (1989) J. Biol. Chem. 264, 9880–9884.
281 Granger, D.M., Hoellwarth, M.E. and Parks, D.A. (1986) Acta Physiol. Scand. Suppl. 548, 47–62.
282 Stirpe, F. and Della Corte, E. (1969) J. Biol. Chem. 244, 3855–3863.
283 Della Corte, E. and Stirpe, F. (1972) Biochem. J. 126, 739–745.
284 Saito, T. and Nishino, T. (1989) J. Biol. Chem. 264, 10015–10022.
285 Saito, T., Nishino, T. and Massey, V. (1989) J. Biol. Chem. 264, 15930–15935.
286 Nishino, T., Nishino, T., Schopfer, L.M. and Massey, V. (1989) J. Biol. Chem. 264, 2518–2527.
287 Misra and Fridovich, I. (1972) J. Biol. Chem. 247, 3170–3175.
288 Wever, R., Oudega, B. and Van Gelder, B.F. (1973) Biochim. Biophys. Acta 302, 475–478.
289 Gotoh, T. and Shikama, K. (1976) J. Biochem. 80, 387–397.
290 Wallace, W.J., Houtchens, R.A., Maxwell, J.C. and Caughey, W.S. (1982) J. Biol. Chem. 257, 4966–4977.
291 Caughey, W.S. and Watkins, J.A. (1985) in Handbook of Methods for Oxygen Radical Research (Greenwald, R.A., ed.), CRC Press, Boca Raton.
292 Lin, W. (1982) Proc. Natl. Acad. Sci. USA 79, 3773–3776.
293 Misra, P.C., Craig, T.A. and Crane, F.L. (1984) J. Bioenerg. Biomemb. 16, 143–152.
294 Halliwell, B. (1978) Planta 140, 81–88.
295 Gross, G.G., Janse, C. and Elstner, E.F. (1977) Planta 136, 271–276.
296 Mäder, M. and Amberg-Fisher, V. (1982) Plant Physiol. 70, 1128–1131.
297 Chalmers, J.D.C., Colman, J.O.D. and Hawes, C.R. (1986) Biochem. Soc. Trans. 14, 734–735.
298 Askerlund, P., Larsson, C., Widell, S. and Møller, I.M. (1987) Physiol. Plant 71, 9–19.
299 Vianello, A. and Macri, F. (1989) Biochim. Biophys. Acta 980, 202–208.
300 Doke, N. (1983) Physiol. Plant Pathol. 23, 345–357.
301 Doke, N. (1983) Physiol. Plant Pathol. 23, 359–367.
302 Doke, N. and Chai, H.B. (1985) Physiol. Plant Pathol. 27, 323–334.
303 Akazawa, T. and Conn, E.E. (1958) J. Biol. Chem. 232, 403–415.
304 Kalyanaraman, V.S., Kumar, S.A. and Mahadevan, S. (1975) Biochem. J. 149, 577–584.
305 Kukreja, R.C., Kontos, H.A., Hess, M.L. and Ellis, E.F. (1986) Circ. Res. 59, 612.
306 Harkin, J.M. and Obst, J.R. (1973) Science 180, 296–298.
307 Stafford, H.A. (1974) Annu. Rev. Plant Physiol. 25, 459–468.
308 Elstner, E.F. and Heupel, A. (1976) Planta 130, 175–180.
309 Westermark, U. (1982) Wood Sci. Technol. 16, 71–78.
310 Wariishi, H. and Gold, M. (1990) J. Biol. Chem. 265, 2070–2077.
311 Chaney, R.L., Brown, J.C. and Tiffin, L.O. (1972) Plant Physiol. 50, 208–213.
312 Sijmons, P.C., Van Den Briel, W. and Bienfait, H.F. (1984) Plant Physiol. 75, 219–221.
313 Bienfait, H.F. (1985) J. Bioenerg. Biomemb. 17, 73–83.
314 Cakmak, I., Van Der Wittering, D.A.M., Marschner, H. and Bienfait, H.F. (1987) Plant Physiol. 85, 310–314.
315 Hendry, G.A.F. and Brocklebank, K.J. (1985) New Phytol. 101, 199–206.
316 Warburg, O. (1908) Hoppe Seylers Z. Physiol. Chem. 57, 1–16.
317 Monroy, A. (1965) Chemistry and Physiology of Fertilization, Holt, Rinehart and Winston, New York.
318 Veron, M., Foerder, C., Eddy, E.M. and Shapiro, B.M. (1976) Cell 10 321–328.
319 Foerder, C.A., Klebanoff, S.J. and Shapiro, B.M. (1978) Proc. Natl. Acad. Sci. USA 75, 3138–3187.
320 Heinecke, J.W. and Shapiro, B.M. (1989) Proc. Natl. Acad. Sci. (USA) 86, 1259–1263.
321 Takahashi, A., Totsune-Nakano, H., Nakano, M., Mashiko, S., Suzuki, N., Ohma, C. and Inaba, H. (1989) FEBS Lett. 246, 117–119.
322 Brooks, S.E. and Schneider, D.L. (1985) J. Protozool. 32, 330–333.
323 Ayers, A.R., Ayers, S.B. and Eriksson, K-E. (1978) Eur. J. Biochem. 90, 171–181.
324 Morpeth, F.F. (1985) Biochem. J. 228, 557–564.
325 Eriksson, K-E., Pettersson, B. and Westermark, U. (1974) FEBS Lett. 49, 282–285.
326 Ayers, A.R. and Eriksson, K-E. (1982) Methods Enzymol. 89, 129–135.
327 Eriksson, K-E. (1981) Ekman Days 1981 Int. Symp. Wood Pulping Chem. 3, 60–65.
328 Sekkat, C., Dornand, J. and Gerber, M. (1988) Immunology 63, 431–437.
329 Dornand, J. and Gerber, M. (1989) Immunology 68, 384–391.
330 Shibanuma, M., Kuroki, T. and Nose, K. (1988) J. Cell. Physiol. 136, 379–383.
331 Marshall, P.J. and Lands, W.E.M. (1986) J. Lab. Clin. Med. 108, 525–534.
332 Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. (1979) Proc. Natl. Acad. Sci. USA 76, 333–337.
333 Henricksen, T., Mahoney, E.M. and Steinberg, D. (1981) Proc. Natl. Acad. Sci. (USA) 78, 6499–6503.
334 Heinecke, J.W., Rosen, H. and Chait, A. (1984) J. Clin. Invest. 74, 1890–1894.
335 Parthasarathy, S. Printz, D.J., Boyd, D., Joy, L. and Steinberg, D. (1986) Arteriosclerosis 6, 505–510.
336 Rankin, S.M. and Leake, D.S. (1987) Biochem. Soc. Trans. 15, 485–486.
337 Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) Proc. Natl. Acad. Sci. USA 81, 3883–3887.