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Dioxygen-Derived Radicals in Biological Systems [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1985 **311**, 605-615
doi: 10.1098/rstb.1985.0167

Phil. Trans. R. Soc. Lond. B **311**, 605–615 (1985)
Printed in Great Britain

Dioxygen-derived radicals in biological systems

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Three instances of the involvement of dioxygen-derived radicals in biological systems are considered. The first concerns the formation of radicals in the haemolytic reactions induced by treatment of erythrocytes by phenylhydrazine, as an example of the so-called 'oxidant drugs'. The evidence for the formation of phenyl radicals is considered and their origin in the oxidation of phenylhydrazine by a ferryl derivative of haemoglobin postulated. The relevance to the formation of phenylated iron and porphyrin species is described. It is suspected that many instances of oxidative damage to cellular systems result from the coincidence of unsequestered redox-active metal ions (particularly those of iron and copper), reductants, and dioxygen. As an example, the damage to hepatocytes, grown in a culture medium containing cysteine, is described. The formation of radical species derived from dioxygen during the respiratory burst associated with phagocytosis is discussed. A new electrochemical method of detecting the superoxide ion produced during the respiratory burst is described. Particular emphasis is placed on the relation between the production of radical species such as the hydroxyl radical and the superoxide ion, and the extent of phagocytosis.

Introduction

A reasonable assumption about the use of dioxygen in biological systems is that unless there is a loss of control in, or damage to, the cell, most reactions involving this molecule and capable of eliciting much free energy proceed without the production of the relevant radical derivatives, i.e. the superoxide ion and the hydroxyl radical (Hill 1981; Greenwood & Hill 1982). Things can go wrong (Halliwell & Gutteridge 1985) when that control is absent: when there is a surfeit of reductant; when the spatial or temporal mislocation of dioxygen, electrons (reductant) and redox-active metal ions such as iron and copper lead to a ready interchange of electrons between the components; or when the components of the defence are deficient, impaired or in disarray. Many of the same features apply to hydrogen peroxide as a thermodynamically vigorous, but kinetically restrained reactant.

A major problem in unravelling the reactions has been the internecine nature of the relations between the species. Thus the following series of reactions are known to occur:

$$O_2 + e \text{ (a variety of reductants)} \rightarrow O_2^-; \quad (1)$$

$$2O_2 + 2H^+ \rightarrow H_2O_2 + O_2; \quad (2)$$

$$H_2O_2 + Fe^{II} \text{ (or } Cu^{I}) \rightarrow OH^{\cdot} + OH^- + Fe^{III} \text{(or } Cu^{II}); \quad (3)$$

$$O_2^- + OH^{\cdot} \rightarrow O_2 + OH^-; \quad (4)$$

$$OH^{\cdot} + H_2O_2 \rightarrow OH^- + HO_2^{\cdot}. \quad (5)$$

In addition, most components of a cell will react with the hydroxyl radical leading to the propagation of chain reactions. There is little doubt that the hydroxyl radical exhibits a

ferocious reactivity, little remaining unscathed in its neighbourhood. Its properties as a very weak acid are often overlooked but are relevant to the ability of its conjugate base, the oxene radical anion, $O^{-\bullet}$, to act as a ligand to metal ions. As D. Dolphin (this symposium) has described, there is very good evidence for such ferryl complexes as intermediates in reactions of cytochrome oxidase, mono-oxygenases and peroxidases:

$$Fe^{III} + H_2O_2 \rightarrow (Fe^{V}O^{2-} \leftrightarrow Fe^{IV}O^{-} \leftrightarrow Fe^{III}O) + H_2O. \quad (6)$$

The reactivity of the superoxide ion is pallid by comparison with that of the hydroxyl radical. Indeed, doubt has been cast (Fee *et al.* 1979; Fee 1982) on the so-called 'toxicity' of superoxide and its malignant role is simply assigned as a precursor of hydrogen peroxide, itself the presumed precursor of the hydroxyl radical. However, an agent that is the ready precursor of a toxic material is also a toxin. Those who dismiss the superoxide as a noxious agent overlook its unusual combination of properties: it is a good, and rapid, one-electron reductant; its acidity is such that in biological media it exists both as a strongly solvated anion, $O_2^{-}(aq)$, and, in its conjugate acid, $O_2H^{\bullet}$, a more reactive and neutral species, which, though solvated, might be able to partition more effectively into non-aqueous phases. Thus, once formed it could be effectively distributed throughout a cell. In addition, if the superoxide ion is formed in a non-aqueous environment, it will have the properties of a strong base. Few species combine these properties; indeed the closest analogues might be considered to be quinones and their reduction products. We can presume that the coincidence in time or space of dioxygen, reductants and metal ions, when one or more of these is uncontrolled, poses a threat to any cell. We shall be concerned with three examples; two involve damage to the cell while in the third the production of these dioxygen-derived species *by* the cell is used to *cause* damage.

Phenylhydrazine-induced haemolysis

There are (Beutler 1978) a number of agents (table 1) that cause haemolysis, especially in those (table 2) with deficiences in, or an impairment of, the cellular apparatus required to maintain the redox state of haemoglobin. The focal point of the reaction is oxyhaemoglobin and, for example, if the haemoglobin is in the carbon monoxy form, the reactions with these agents proceed quite differently or not at all. All the reagents react with oxyhaemoglobin freed from the cell with essentially the same dramatic consequences (table 3). A century ago, it was observed (Hoppe-Seyler 1885) that phenylhydrazine caused the haemolysis of erythrocytes and this reaction has served as a model for drug-induced haemolysis ever since. One of the results of treating erythrocytes with phenylhydrazine and like materials is the formation of methaemoglobin, which has given rise to the inappropriate description of such agents as 'oxidant drugs'; inappropriate because they are all reducing agents with respect to any form of haemoglobin other than deoxyhaemoglobin. A number of small molecular products arising from the reaction of phenylhydrazine with erythrocytes have been identified (Shetlar & Hill 1985) and others are suspected. Those identified include benzene, nitrogen, hydrogen peroxide, the superoxide anion and the phenyl radical; those suspected are phenyldiazene, and the phenylhydrazyl and phenyldiazenyl radicals.

We (Hill & Thornalley 1981, 1982, 1983) have been principally concerned with the identification of the phenyl radical. It is easily intercepted by the use of a number of spin traps. These, usually nitrones or nitroso derivatives, react (Janzen 1980) with radicals to give more

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Table 1. Some agents that cause haemolysis

antimalarials such as primaquine and quinine
antipyretics and analgesics such as aspirin
sulphones such as 4,4′-diaminodiphenylsulphone
hydrazines such as acetylphenylhydrazine

Table 2. Conditions that give rise to enhanced sensitivity to oxidative damage to red cells

catalase deficiency
vitamin E deficiency
decreased NADPH production
abnormal glutathione metabolism
paroxysmal nocturnal haemoglobinuria
erythropoietic protoporphyria
thalassaemia syndromes
sickle-cell anaemia

Table 3. events associated with phenylhydrazine-induced haemolysis

blood assumes a brown–green coloration
haemoglobin forms 'green' haemoglobin in which the haem group is modified
destabilization of the globin portion leads to denaturation and precipitation
protein–protein cross-linking occurs within the supporting network
lipid peroxidation occurs within the membranes
oxyhaemoglobin forms methaemoglobin

stable nitroxides. The latter have characteristic electron paramagnetic resonance spectra which can be used both to detect and, in favourable cases, characterize the nitroxide and thus the reactive radical precursor. The production of the phenyl radical provides just such a favourable case; the product of the reaction with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) is stable such that it can be isolated and characterized by mass spectrometry. It gives rise to a six-line signal in the e.p.r. spectrum, the unpaired electron coupling to [^{14}N] ($I = 1$; three lines) of the nitroxide group, each of which is also coupled to the neighbouring C[^{1}H] ($I = \frac{1}{2}$; two lines). The superoxide anion had earlier been identified (Goldberg & Stern 1975) as a product. It was not detected in the spin-trapping experiments, but that is not surprising given its slow rate of reaction with most spin traps. Both water-soluble and lipid-soluble spin traps react with phenyl radicals though the latter seem to be more effective (figure 1). Certainly, they are better able to *protect* the erythrocytes from haemolysis. In a sense, it appears that there is an *endogenous* trap for the phenyl radical within the erythrocyte; the *iron* of haemoglobin! There is now (Kunze & Ortiz de Montellano 1983) convincing evidence that complexes containing the phenyl radical directly attached to the iron of haemoglobin are formed during the reaction. The η_1-$C_6H_5Fe^{III}$ myoglobin has been isolated (Ringe *et al.* 1984) and its structure determined by X-ray diffraction. Recent reports on analogous complexes of iron porphyrins have provided support (Mansuy *et al.* 1982; Battioni *et al.* 1983) for the proposal that this organometallic derivative is the precursor of the *N*-phenylated and *C*-phenylated porphyrins that are the cause of the green and blue compounds formed in the reaction of phenylhydrazine with haemoglobin or erythrocytes. It is therefore possible that the phenyl radical formed within the haem pocket can react with the iron or escape; if the former, the subsequent interaction with oxygen can lead to reaction at the nearby pyrrole nitrogen or, perhaps, at the *meso* position of the porphyrin.

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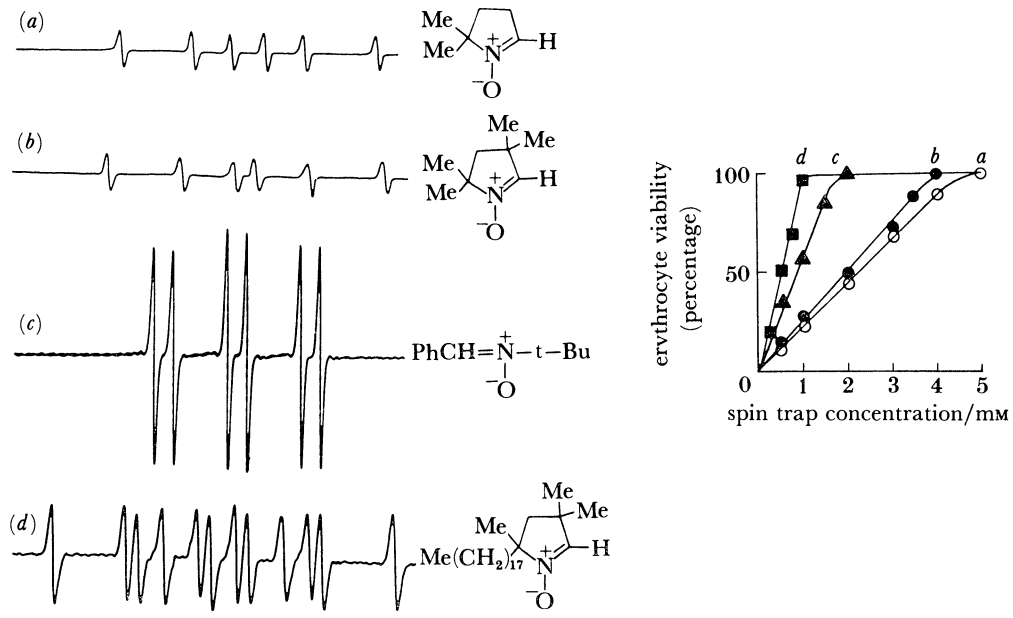

FIGURE 1. (*a*)–(*d*) shows the e.p.r. spectra of the spin adducts formed in the presence of erythrocytes, phenylhydrazine, and the indicated spin traps. The erythrocyte viability in the presence of the spin trap and hydrazine is shown in the inset. (Adapted from Hill & Thornalley 1983.)

If it escapes, it can abstract a hydrogen from a multitude of sources. However, it is a relatively long-lived radical and its mean free path might allow it to reach the cell membrane, there to initiate radical chain reactions. In the discussion of radical damage in biological systems, much emphasis has reasonably been placed on the most reactive radicals, such as the hydroxyl radical. However, it is possible that, because the latter will react with compounds very close to its site of formation, it might lead to damage that is inconsequential or easily repaired. The longer-lived radicals may cause more harm by reaching parts of the cell where damage is not easily repaired or where other reactants, like dioxygen, are available to amplify the damage.

How is the phenyl radical formed? It has been proposed (Goldberg *et al.* 1976) that ferrylhaemoglobin is formed during the reaction of haemoglobin with hydrogen peroxide formed in the erythrocyte, or by reduction of dioxyhaemoglobin by phenylhydrazine, in a manner analogous to the formation of ferryl intermediates in reactions of peroxidases or cytochrome P_{450}:

$$HbFe^{III} + H_2O_2 = HbFe^{III}OOH + H^+; \quad (7)$$

$$HbFe^{III}OOH = HbFe^{V}O + OH^-. \quad (8)$$

The ferryl intermediate, $HbFe^{V}O$, can then form the $HbFe^{IV}O$–porphyrin radical cation which may, by subsequent reaction with phenylhydrazine, give the phenylated haem derivatives, or it may react directly with phenylhydrazine to give a phenyldiazene complex:

$$HbFe^{V}O + PhNHNH_2 = HbFe^{III}{:}PhN{=}NH + H_2O. \quad (9)$$

The latter could rearrange to give the phenyldiazenyl radical adduct as a precursor to the formation of the phenyl radical:

$$HbFe^{III}{:}PhN{=}NH = HbFe^{II}{:}PhN{=}N^{\cdot} + H^+. \quad (10)$$

Loss of dinitrogen leads to the formation of Hb^{II} and the phenyl radical,

$$HbFe^{II}:PhN{=}N^{\cdot} = HbFe^{II} + N_2 + Ph^{\cdot}. \quad (11)$$

The latter may escape from the haem pocket or it may be trapped by the iron to form the phenylated derivative:

$$HbFe^{II} + Ph^{\cdot} = HbFe{-}Ph. \quad (12)$$

Subsequent decomposition of this organometallic derivative in the presence of dioxygen would lead to the formation of the phenylated products of the haem. The identification of the latter (Ortiz de Montellano & Kunze 1981; Saito & Itano 1981; Augusto *et al.* 1982), together with the characterization of the iron–phenyl derivatives, have provided much greater insight into the mechanism of this complex process.

There are two features that may be unique to the reactions of phenylhydrazine: its ability to act as both a multi-electron *and* multi-proton donor. In addition, its oxidation product, phenyldiazene, can be considered to be isoelectronic with dioxygen, which perhaps aids its entry to the haem site or even allows bonding to the iron. It should not therefore be concluded that insight derived from a study of the mechanism of phenylhydrazine-induced haemolysis may necessarily be relevant to the reactions of the other 'oxidant drugs'.

Radical damage to hepatocytes grown in culture

The coincidence of reducing agents, redox-active metal ions and dioxygen is a far from infrequent occurrence. Many media capable of supporting the growth of organisms in culture provide just such an opportunity. Common constituents of culture media are thiols; these are ready reductants and metal-sequestrants. In the presence of dioxygen, the autoxidation of, for example, cysteine yields hydrogen peroxide and various oxidized derivatives, including cysteine sulphinic and sulphonic acids and cystine (Benesch & Benesch 1955; Cavallini *et al.* 1969; Jocelyn 1972; Zwant *et al.* 1981). With the use of spin traps, it has been shown (Saez *et al.* 1982) that thiyl radicals derived from cysteine are formed under conditions appropriate to the growth of hepatocytes. This, perhaps, is not surprising. However, the spin adduct derived from the hydroxyl radical is also present and this is confirmed by the identification of the adduct formed by trapping the 1-hydroxylethyl radical when the autoxidation is effected in the presence of ethanol:

$$OH^{\cdot} + CH_3CH_2OH = H_2O + CH_3CH^{\cdot}(OH). \quad (13)$$

The relative amounts of the radical adducts depend on the cysteine concentration, on chelating agents or iron(III) or copper(II) salts added to the system, and on the concentration of the spin trap. The addition of superoxide dismutase alone stimulates the production of the two radicals, while catalase inhibits the formation of the radicals. This suggests that hydrogen peroxide is formed by the disproportionation of the superoxide ion, the latter remaining undetected because of its low rate of reaction with the spin trap. It is suggested that thiyl radicals ($RS^{\cdot}$), formed initially by the oxidation of cysteine by iron(III) or copper(II), react with dioxygen to give oxidized cysteine derivatives or with more cysteine to give reduced cystine:

$$RS^{\cdot} + RSH = RSSR^{-\cdot} + H^{+}. \quad (14)$$

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This species may react with dioxygen to give the superoxide ion:

$$RSSR^{-\cdot} + O_2 = RSSR + O_2^{-}. \quad (15)$$

The latter may then form hydrogen peroxide and, if the Fenton reaction ensues, the hydroxyl radical. In addition, it is possible that the hydroxyl radical may be formed by the direct reaction of $RSSR^{-\cdot}$ with hydrogen peroxide:

$$RSSR^{-\cdot} + H_2O_2 = RSSR + OH^{-} + OH^{\cdot}. \quad (16)$$

The effect of autoxidizing cysteine on the hepatocytes is to cause the loss of most of their ATP and glutathione with an increase leakage of lactate dehydrogenase, presumably a result of oxidative damage to membrane lipids. The conditions used in the experiments were far from unusual, and thus care is advised in the culture of cells in media prone to autoxidation.

Production of oxygen-derived species by leukocytes

Leukocytes (in particular neutrophils and macrophages) show (Baehner *et al.* 1982) a marked increase in their use of oxygen when engaging in phagocytosis. This respiratory burst is elicited by a number of agents (table 4). The oxygen thus used leads to the formation of a variety of products. There is good evidence that the first product is superoxide, derived ultimately from the oxidation of NADPH. It was first identified as a product through the reduction of exogenous cytochrome *c*. We were concerned to find more direct methods of detecting this product. To that end, we first used (Green *et al.* 1979) the technique of spin trapping, though this is not a particularly effective method for superoxide because most spin traps react slowly with it, at least compared with their rates of reaction with other radicals. The superoxide was, however, detected under the following circumstances (Green *et al.* 1979; Hill & Okolow-Zubkowska 1981; Okolow-Zubkowska & Hill 1981): activation of the respiratory burst with the use of phorbol myristate actate; in the presence of azide ion; and at low temperatures (22 °C). We have recently found (Green *et al.* 1984) that it is possible to exploit the electrochemical oxidation of superoxide to detect the production of this species. An 'opsonized' electrode was prepared (figure 2) that had IgG adsorbed on the electrode surface. With the potential poised at a value that would oxidize any superoxide produced, neutrophils were placed in solution. The current that resulted could be inhibited by superoxide dismutase, and passed for a time consistent with

Table 4. Some agents capable of stimulating the respiratory burst in polymorphonuclear leukocytes

complement
phospholipase *c*
phorbol myristate acetate
formalin-treated red blood cells
C_3- or IgG-coated micro-organisms or latex particles
lipopolysaccharide C_3-coated paraffin oil droplets
C_3-coated zymosan (yeast cell wall)
digitonin and saponin (detergents)
ionophores such as A23187
antineutrophil antibodies
concanavalin A (lectin)
cytochalsin E
endotoxin

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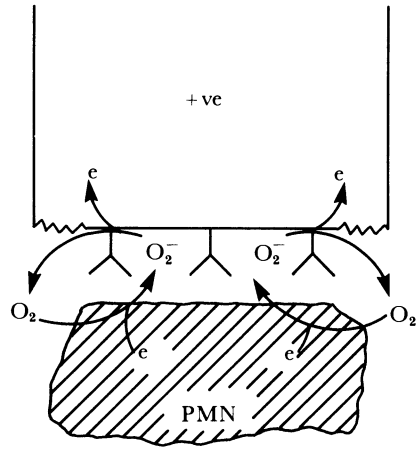

FIGURE 2. A scheme showing the formation of the superoxide ion by neutrophils (PMN) at an electrode surface modified with IgG. On formation, the superoxide is re-oxidized at the electrode.

it being associated with the respiratory burst. It was not elicited by, for example, bovine serum albumin, nor was it affected by the presence of catalase. It was straightforward to show that it was being produced *at* the electrode surface. Approximately 10% of the oxygen taken up gave rise to superoxide thus detected. Given the rapid disproportionation of superoxide, this was considered a reasonable level of detection. Recent results (H. A. O. Hill *et al.* 1985) have shown that, by using an electrode with dimensions not dissimilar to those of a neutrophil, it is possible to detect the superoxide produced by a single cell.

The superoxide anion is formed; is its formation associated with damage to the target organism? Its toxicity is now thought to reside in its role as a precursor of more lethal species, and thus interest centres on other oxygen-derived species, especially hydrogen peroxide and the hydroxyl radical. Of the presence of the former there is no doubt; it presumably arises through the disproportionation of the hydroperoxyl radical:

$$HO_2 + HO_2 = O_2 + H_2O_2. \qquad (17)$$

Whence the hydroxyl radical? It has been detected (Clifford & Repine 1984) by a number of methods, including spin trapping. None of these methods are completely secure, though the evidence (table 5) is more than circumstantial. (The use of spin traps readily leads to the detection of the product associated with the formation of $OH^{\cdot}$. It is possible (Finkelstein *et al.* 1980) that the same product can be formed, albeit more slowly, by the decomposition of the product formed from the adduct of DMPO with HO_2. It has been stated (Halliwell 1984) that the absence of inhibition by hydroxyl radical scavengers such as formate and ethanol indicated that hydroxyl radicals are not formed during the respiratory burst. However, these scavengers were *not used* (Green *et al.* 1979), because they inhibit the respiratory burst. Mannitol, which at the concentrations used does not inhibit the respiratory burst, reduced the yield of the hydroxyl radical adduct of DMPO by 50%. The purpose of using spin traps is to allow the detection of radicals under conditions where the normal functions of the cell *are not perturbed*.) The hydroxyl radical can be formed via the Fenton reaction

$$H_2O_2 + Fe^{II} = OH^{\cdot} + OH^- + Fe^{III}; \qquad (18)$$

Table 5. A summary of evidence for the formation of superoxide and hydroxyl radicals during the respiratory burst associated with phagocytosis

observations	inferences
superoxide dismutase (SOD inhibits the production of ethylene from methional	hydroxyl radicals arise from an O_2^--dependent reaction
SOD inhibits the production of DMPO(OH) from DMPO	
SOD inhibits the reduction of cytochrome *c* by stimulated neutrophils	O_2^- is produced by stimulated cells
catalase only slightly reduces the yield of DMPO(OH)	catalase is not as effective an inhibitor as SOD, suggesting that H_2O_2 is not the immediate precursor of $OH^{\cdot}$
catalase has only a slightly inhibitory effect on the generation of ethylene from methional	
hydroxyl radical scavengers, e.g. mannitol, inhibit the formation of DMPO(OH)	hydroxyl radicals are formed during the respiratory burst
hydroxyl radical scavengers, e.g. benzoic acid, ethanol, or mannitol, inhibit the formation of ethylene from methional	
phorbol myristate acetate (PMA)-stimulated cells yield DMPO(OOH) from DMPO	PMA-soluble stimulus results in the release of specific granules only; not the azurophic granules which contain MPO
both DMPO(OH) and DMPO(OOH) are formed from DMPO in the presence of azide	azide inhibits myeloperoxidases, thereby preventing formation of hypochlorite

but what is the source of the iron? Lactoferrin, present in the neutrophils and released during the respiratory burst, may be a source of iron, but the ability of this protein to release iron within the phagocytic vacuole, which is crucially dependent on its state of repletion, has not yet been confirmed. The formation of the superoxide adduct of DMPO, is increased relative to that of the hydroxyl radical in the presence of azide. The latter inhibits myeloperoxidase, which catalyses the reaction:

$$H_2O_2 + Cl^- = OCl^- + H_2O. \quad (19)$$

This leads to the suggestion (Okolow-Zubkowska & Hill 1981) that the following reaction might, in part, also play a role in the formation of $OH^{\cdot}$:

$$O_2^- + HOCl = OH^{\cdot} + O_2 + Cl^-. \quad (20)$$

One difficulty in designating a single species as the toxic entity is the superfluity of reactions between the oxygen-derived products. Thus,

$$HOCl + H_2O_2 = {}^1O_2 + H_2O + HCl \quad (21)$$

and

$$Cl^- + OH^{\cdot} = Cl^{\cdot} + OH^- \quad (22)$$

add to the complexity of the situation. Of course, it is possible that this complexity is exploited in the sense that the reduction of dioxygen in the presence of chloride, iron and the appropriate catalysts leads to a range of highly reactive species, of which at least one will prove lethal to any given cell. The reduction of dioxygen in the respiratory burst is an essential part of the host defence, as witnessed by the consequences of the absence of this facility in those suffering

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

from chronic granulomatous disease. A deficit of myeloperoxidase does not appear to lead to such devastating effects.

The exploitation of such reactive chemistry cannot be without risk. In 'normal' circumstances the dioxygen-derived products are presumably directed towards the target cell; some of the consequences are described in table 6. If some other physiological function of the host is

TABLE 6. CONSEQUENCES OF PRODUCTION OF DIOXYGEN-DERIVED SPECIES DURING THE RESPIRATORY BURST

cell lysis
synthesis of *N*-chloramines
transformation of prostaglandins
methaemoglobin formation in erythrocytes
degradation of hyaluronic acid
bacterial mutagenesis

impaired, it is possible that this array of reagents may be misplaced. It may therefore be important that the leukocyte membrane and the cell wall of the target are contiguous and even that the target is fully contained within the phagocytic vacuole. If that does not happen, the radical species will be able to reach the host tissues. We have found (M. J. Green, H. A. O. Hill & D. G. Tew, unpublished observations) that the ability to detect the superoxide anion, by using the reduction of cytochrome *c* as a monitor, does not correlate with oxygen utilization by neutrophils at low target:cell ratios. It is possible that cytochrome *c* is able to intercept only that fraction of superoxide that escapes from the neutrophil–target boundary interface. At high target:cell ratios, imperfectly formed interfaces may be more numerous and therefore the amount of superoxide detected increases. Many experiments using, for example, IgG-coated latex particles are performed at such high ratios; they might therefore have more relevance to situations *in vivo* where the neutrophils are similarly 'overloaded'. It will be interesting to observe if the quantity of radicals that can so 'escape' is related, not only to the target:cell ratio but also to the ease with which the two surfaces can be made contiguous. This may depend on the surface topography and target shape.

CONCLUSIONS

The accidental coincidence of dioxygen, electrons and redox-active metal ions must be avoided if deleterious effects are not to ensue. The intrusion of exogenous materials, not subject to enzymatic controls, can lead to the production of noxious species that require action by an elaborate defensive system to restrict damage. If this defensive apparatus is absent, attenuated or overcome, then cellular death will follow. In phagocytosis by neutrophils or macrophages, the positions are reversed and the attack, exploiting dioxygen-derived species, is mounted by bringing together the precursors: dioxygen, NADPH, and as-yet uncharacterized metal ions. Oxidation products derived from chloride are added for good measure. It is possible that there is an element of control of the disposition of these attacking forces that is at present unidentified, such that damage to the launching cell is minimized. Also, it is not yet known which of the reactive species is responsible for the death of the target cell, nor are the events that lead to this terminal event recognized. What is certain is that of all reagents exploited by living systems, dioxygen carries with it the greatest risk, yet bestows the greatest benefit.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

I am grateful to Dr J. V. Bannister, Dr M. J. Green, Dr P. J. Thornalley and Mr D. G. Tew for permission to describe their work. I thank Professor M. D. Shetlar for much profitable discussion. I am glad to acknowledge the support of the British Heart Foundation.

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Discussion

Catherine Rice-Evans (*Department of Biochemistry, Royal Free Hospital School of Medicine*). Does the phenylhydrazyl radical interact with the iron ion in the haem group in the same way as Dr Hill described for the phenyl radical? Is there any evidence that radicals derived from unsubstituted hydrazine, for example, the hydrazyl or diazyl radical, interact in the same way with the haem iron?

H. A. O. Hill. There is no *direct* evidence that nitrogen-centred radicals react in the same way with the iron in the haem group. It is possible, however, that such interactions play an important part in the mechanism (Shetlar & Hill 1985).

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