Free-radical generation by copper ions and hydrogen peroxide
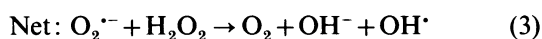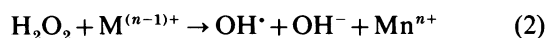
Stimulation by Hepes buffer

Jeremy A. SIMPSON,* Kevin H. CHEESEMAN, Susan E. SMITH and Roger T. DEAN
Department of Biology and Biochemistry, Brunel University, Uxbridge UB8 3PH, U.K.

Hydroxyl radicals (OH·), generated by a phosphate-buffered Cu^{2+}/H_2O_2 system, were detected by lucigenin-amplified chemiluminescence, deoxyribose degradation and benzoate hydroxylation. In each system the buffer, Hepes, was found to stimulate radical generation significantly. There are two main reasons for this effect: Hepes increases Cu^{2+} solubility in phosphate-buffered systems, and forms a complex with Cu^{2+} that is effective in generating OH· from H_2O_2. Pipes, a structurally similar buffer, and histidine, a known Cu^{2+} chelator, were found to have a similar effect. These data suggest that the crucial factor in such free-radical-generating systems is the availability of Cu^{2+}, and that these actions of Hepes should be considered in the design of studies utilizing such systems.

INTRODUCTION

There is currently much interest in the role of free radicals, and especially oxygen free radicals, in human disease (Slater, 1972; Slater, 1984; Clark et al., 1985; Sies, 1985). Superoxide radicals ($O_2^{·-}$) and H_2O_2 are known to be produced in many biological systems, but these are relatively harmless as they react with biomolecules at low rates and specific enzymes exist to remove them. In the presence of certain metal ion complexes, however, the presence of $O_2^{·-}$ and H_2O_2 can lead to the formation of the highly reactive and damaging hydroxyl radical (OH·); for this reason, cells usually keep metal ions very firmly bound in unreactive forms (Halliwell & Gutteridge, 1986). The most prominent reactions involved in this metal-catalysed OH· generation are:

$$O_2^{·-} + M^{n+} \rightarrow O_2 + M^{(n-1)+} \quad (1)$$

$$H_2O_2 + M^{(n-1)+} \rightarrow OH· + OH^- + Mn^{n+} \quad (2)$$

$$\text{Net: } O_2^{·-} + H_2O_2 \rightarrow O_2 + OH^- + OH· \quad (3)$$

The overall reaction (3) is often referred to as the metal-catalysed Haber–Weiss reaction. This has been most often studied with iron salts but copper ions will also catalyse the reaction; in fact cuprous salts will generate OH· from H_2O_2 faster than ferrous salts (Halliwell & Gutteridge, 1984). If cupric salts (as used in the present paper) are mixed with H_2O_2 the following reaction occurs:

$$Cu^{2+} + H_2O_2 \rightarrow Cu^+ + HO_2· + H^+ \quad (4)$$
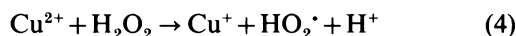
The Cu^+ ions thus generated are able to generate OH· via reaction (2).

In the body free copper ions are not normally available but are bound tightly to serum albumin or incorporated into caeruloplasmin. The tendency of copper ions to bind readily to amino groups of proteins has often made it appear that proteins will thus prevent copper-ion-dependent OH· formation. Actually, the OH· generation is not prevented but rather localized to the site of binding

of the copper ions, and the protein molecule itself will be damaged by the OH· radicals (Gutteridge & Wilkins, 1983; Hunt et al., 1988). Histidine residues have been particularly implicated as sites of copper-ion-dependent protein degradation (Dean, 1987). In fact, the effect of complexing catalytic metal ions on OH· production is not readily predictable. Chelating agents may promote metal-ion-dependent OH· formation (e.g. Fe^{3+}–EDTA) or inhibit it (e.g. Fe^{3+}–desferal), depending on a number of factors including (i) the solubility of the complex, (ii) the redox potential of the $M^{(n-1)+}/M^{n+}$ couple and consequently its ability to be reduced by $O_2^{·-}$ and (iii) whether or not it has a free co-ordination site and so is able to catalyse H_2O_2 breakdown (Graf et al., 1984).

In this paper we have studied the Cu^{2+}/H_2O_2-dependent generation of chemiluminescence (CL), deoxyribose degradation and benzoate hydroxylation, which, used in conjunction, can be taken as markers of OH· production. Other oxidative species may be involved to some degree (Johnson et al., 1985), particularly in the CL assay (Muller-Peddinghaus, 1984). All of these parameters are markedly stimulated by the presence of the commonly used buffer Hepes and we suggest that this is due to a Cu^{2+}–Hepes interaction.

MATERIALS AND METHODS

Reagents

All chemicals were of the highest purity available and were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Solutions of H_2O_2 were made up fresh as required. Stocks of reagents were always made up 10-fold the final concentration required and kept on ice until use.

Radical generation

In most cases free radicals were generated by H_2O_2 (5 mM) and $CuSO_4$ (0.5 mM) in a 10 mM-sodium phosphate (pH 7.2)-buffered system at room temperature. All test compounds, which were adjusted to pH 7.2, were present

---

Abbreviations used: DTPA, diethylenetriaminepenta-acetic acid; CL, chemiluminescence.
* To whom correspondence should be addressed.

from the start of the experiment (before radical genera-
tion). In some experiments the reactant concentrations
were varied as stated in the Figure legends. The following
indices of OH˙ production were used.

(a) CL assay. CL was amplified and detected by the
oxidation of lucigenin (Campbell et al., 1985). A 0.6 ml
volume of 16.7 mM-sodium phosphate buffer, pH 7.2,
containing an appropriate concentration of Hepes was
placed in a clean glass scintillation vial. Then 0.2 ml of
lucigenin (770 μM) was added and the CL baseline was
measured. This was followed sequentially by 0.1 ml each
of 50 mM-H_2O_2 and 5 mM-$CuSO_4$. CL was detected at
room temperature in a liquid-scintillation counter in the
'out-of-coincidence' mode.

(b) Deoxyribose degradation. With the use of the
radical-generating system described above deoxyribose
degradation was carried out essentially as described by
Halliwell & Gutteridge (1981) at 25 °C. Portions (0.9 ml)
of the reaction mixture (described above) containing
10 mM-deoxyribose were taken, and the radical genera-
tion was stopped by the addition of 0.1 ml of 50 mM-
EDTA. These solutions were heated for 10 min at 100 °C
with 0.5 ml of 2.8 % (w/v) trichloroacetic acid and 0.5 ml
of 1 % (w/v) thiobarbituric acid in 50 mM-NaOH, cooled
briefly, and the absorbance was read at 532 nm. In many
instances, when the various chelating agents and buffers
were tested (Fig. 4), turbid solutions formed during the
thiobarbituric acid test. This was overcome by adding
0.1 ml of 10 M-NaOH after cooling the solutions
and then reading at the new absorbance maximum of
545 nm.

(c) Benzoate hydroxylation. With the use of the radical-
generating system described above, hydroxylation of
benzoate (1 mM) was determined as described by Gut-
teridge (1987). Fluorescent products were determined by
excitation at 308 nm with emission at 410 nm in an
LS-3 fluorescence spectrometer (Perkin–Elmer, Beacons-
field, Bucks., U.K.). Results are expressed as the change
in relative fluorescence intensity units with time.

All data presented are representative of at least three
separate experiments.

RESULTS

(1) Hepes-enhanced CL generated by Cu^{2+}/H_2O_2

With the standard conditions of 5 mM-H_2O_2, 0.5 mM-
$CuSO_4$ and 10 mM-phosphate buffer, the presence of
Hepes was an absolute requirement for the generation of
a CL response, but its effect was biphasic (Fig. 1). Below
0.5 mM-Hepes no response was seen, between 0.5 and
2.5 mM a concentration-dependent response was seen,
and higher concentrations (> 10 mM) inhibited the
response. Neither Cu^{2+} nor H_2O_2 alone, with or without
Hepes, induced a response. Therefore, at low con-
centrations, Hepes seemed to be facilitating the reaction
by keeping Cu^{2+} in solution and/or facilitating copper
cycling in the redox potential reaction.

The effect of Hepes was also tested in the absence of
sodium phosphate (Fig. 2). Again Cu^{2+} alone did not
catalyse a CL response. With the addition of Hepes (as
well as Cu^{2+}) a CL response was obtained that was of
much greater intensity, reached a maximum more rapidly
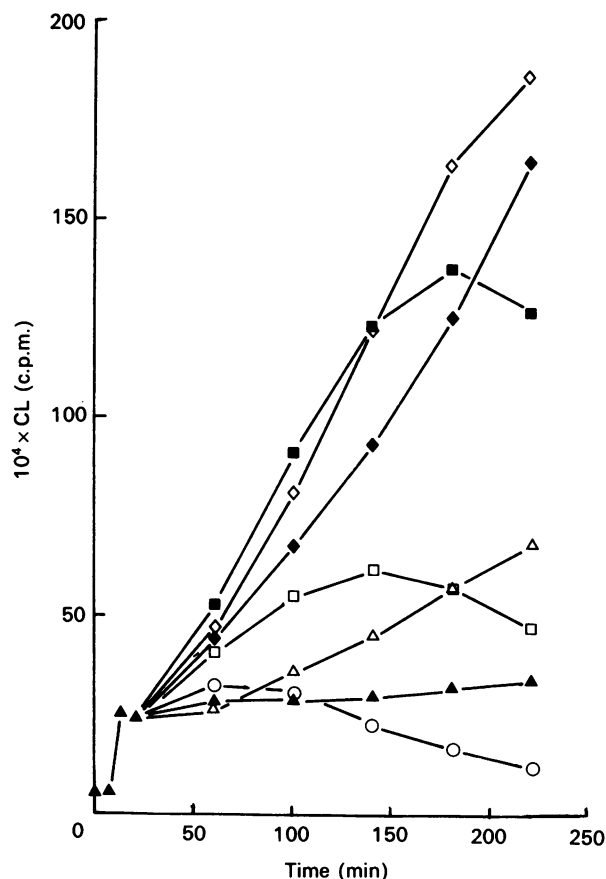and was of shorter duration than that which had been

Fig. 1. CL response of 5 mM-H_2O_2 and 500 μM-$CuSO_4$ in
10 mM-sodium phosphate buffer, pH 7.2, in the presence
of various concentrations of Hepes buffer, pH 7.2

The concentrations of Hepes buffer were 0 μM (▲), 500 μM
(△), 1 mM (◆), 2.5 mM (◇), 5 mM (■), 10 mM (□) and
20 mM (○).

obtained in the presence of phosphate. The response
showed a biphasic dependence on concentration: low
concentrations (2.5–10 mM) stimulated the response in a
dose-related fashion, and high concentrations (20 mM)
inhibited it completely. After the spontaneous termina-
tion of CL the response could be regenerated by the
addition of H_2O_2 but not by any of the other system
components. In this system (with no phosphate present)
Cu^{2+} remains in solution, and yet Hepes (at low
concentrations) still stimulated the response and so can
be presumed to act at least in part, by interaction with
Cu^{2+} and subsequent generation of a more redox-active
complex. High concentrations of Hepes inhibited the
response to a similar degree as in the system with
phosphate present.

The response stimulated by 4 mM-Hepes was influenced
by varying the phosphate concentration over the 1–
100 mM range (Fig. 2). The effect of phosphate did not
show a simple concentration-dependency. Above the
'standard' conditions of 10 mM-phosphate a biphasic
effect was seen: increasing the concentration up to 100 mM
enhanced the CL response proportionally; concentra-
tions above 100 mM were strongly inhibitory. However,
decreasing the phosphate concentration to 1 mM also
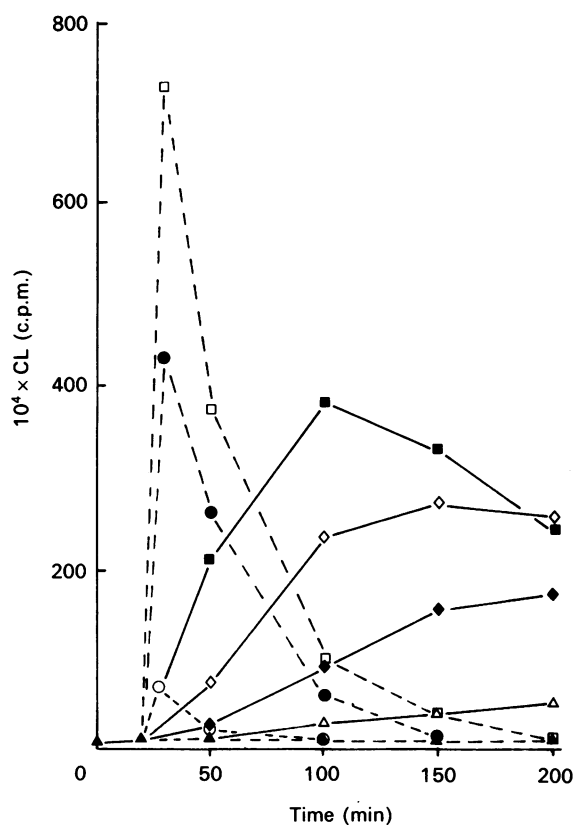inhibited CL. The inhibitory effect of high phosphate

Fig. 2. CL response of 5 mM-H_2O_2, 500 μM-$CuSO_4$ and 4 mM-Hepes buffer, pH 7.2, in the presence of various concentrations of sodium phosphate buffer, pH 7.2

The concentrations of phosphate buffer were 0 mM (●), 1 mM (△), 10 mM (◆), 50 mM (◇), 100 mM (■) and 500 mM (▲). Broken lines represent the response in the absence of phosphate with Hepes at 0 mM (▲), 2.5 mM (□), 4 mM (●) and 10 mM (○). For clarity, we have deliberately used the symbols ▲ and ● twice.

(2) Detection of enhanced radical production by assay of deoxyribose degradation

The phosphate-buffered Cu^{2+}/H_2O_2-induced degradation of deoxyribose was markedly stimulated by the addition of Hepes at 4 mM (Fig. 3), and the production of thiobarbituric acid-reactive material was found to be approximately linear with time for at least 3 h of incubation at 25 °C. We next tested Hepes in this system with a fixed incubation time (1 h) and at concentrations of 0.5, 2.5 and 10 mM (Fig. 4) for direct comparison with the CL assay. At 0.5 mM-Hepes there was no significant stimulation of deoxyribose degradation over control values, but at 2.5 mM the production of thiobarbituric acid-reactive material was increased some 3-fold. In the absence of phosphate buffer the stimulatory effect of Hepes was even more pronounced, being 5-fold over control values with 2.5 mM-Hepes. Other related buffers (Pipes, Mops) and two chelating agents (EDTA and DTPA) were also tested under these conditions. The stimulatory effect of Pipes was very similar to that of Hepes, but the effect of Mops was very much weaker. EDTA and DTPA both inhibited the reaction; indeed, EDTA was used to terminate the reaction throughout. These data are entirely consistent with the CL data.

(3) Benzoate hydroxylation

In a similar way to the deoxyribose degradation, 4 mM-Hepes stimulated some 3-fold the Cu^{2+}/H_2O_2-induced hydroxylation of benzoate in a phosphate-buffered system (Fig. 3). In contrast with the CL and deoxyribose degradation data there was a detectable control reaction; this could be due to the relative sensitivities of the systems and/or a similar (to Hepes) promotory effect of benzoate on radical generation. In the absence of phosphate but the presence of Hepes the rate of hydroxylation over the first 30 min was 6-fold greater than the control system containing neither phosphate nor Hepes, but the reaction slowed down quickly (results not shown).

concentrations, probably by radical scavenging, was not surprising; the other effects, at lower concentrations, were unexpected (see the Discussion section).

With the use of the standard Hepes (4 mM) and phosphate (10 mM) concentrations the effect of varying the Cu^{2+} concentration was studied. The CL response was dose-related up to 50 μM, above which a plateau in the response was obtained and therefore less than 10% of the normal concentration of added Cu^{2+} was necessary. In the absence of Hepes no CL response was obtained even with concentrations of Cu^{2+} up to 5 mM.

The effect on CL of a known Cu^{2+} chelator, histidine, as well as some other 'Good' buffers, were also studied (results not shown). In the phosphate-buffered system histidine (0.5 mM) could replace Hepes, producing the same sort of progressive CL response as the latter, i.e. the type of response shown in Fig. 1. In the presence of Hepes histidine enhanced the CL response at low concentrations (0.05 mM and 0.1 mM), but at high concentrations (1–3 mM) had an inhibitory effect. Pipes behaved in exactly the same manner as Hepes, but Mops did not stimulate the response at any of the concentrations tested (10 μM–20 mM).

DISCUSSION

Hepes in radical-generating (Grootveld & Halliwell, 1986) and CL (Easmon et al., 1980) systems is normally considered a radical scavenger (Hicks & Gebicki, 1986). This is certainly true at the high concentrations used in those systems and in our experiments, but we find stimulatory effects at relatively low concentrations. In our CL systems a certain amount of Hepes was actually necessary to stimulate a response. However, at high concentrations Hepes showed inhibitory effects, which are probably due to its aforementioned radical-scavenging properties. Since Hepes stimulated radical generation, as judged by all the detection systems, in a similar manner, this effect seems to be a genuine increase in the production of OH· rather than an artefact of an individual test system.

The requirement for Hepes in the systems tested indicates a Cu^{2+}–Hepes interaction. We demonstrated, by absorption spectroscopy, the formation of a Cu^{2+}–Hepes complex. Cu^{2+}–phosphate and Cu^{2+}–Hepes complexes yielded absorption spectra with broad peaks at 242 nm and 230 nm respectively (which were not detectable when each of the individual components was tested alone). The peak of the Cu^{2+}–Hepes complex was undetectable when
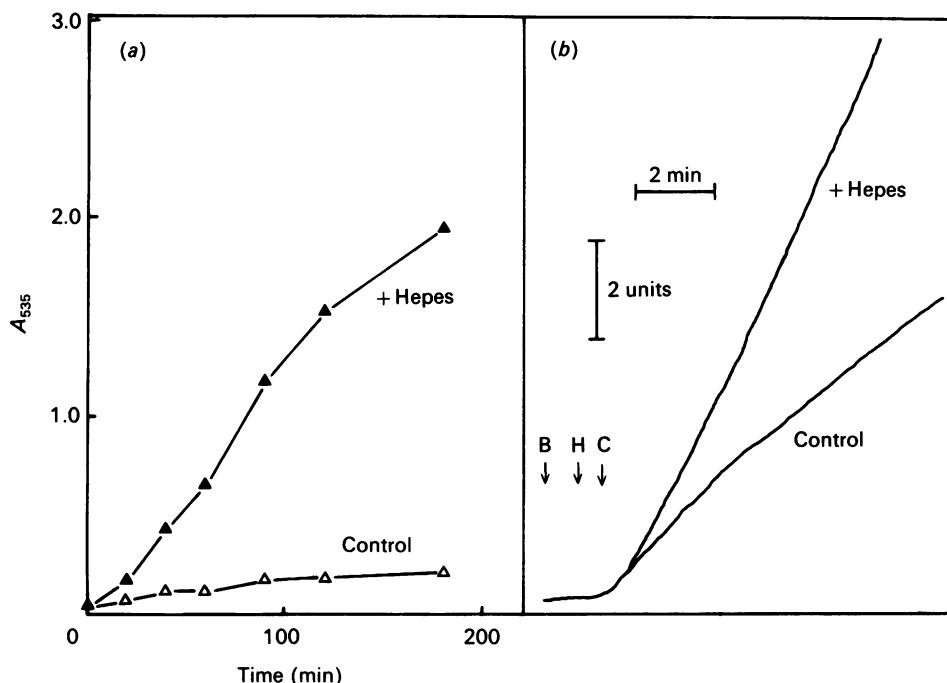
Fig. 3. Effects of 4 mM-Hepes buffer on (a) deoxyribose (10 mM) degradation and (b) benzoate (B) (1 mM) hydroxylation induced by H_2O_2 (H) (5 mM) and $CuSO_4$ (C) (500 μM) in 10 mM-sodium phosphate buffer, pH 7.2

In each case the S.D. was less than 5 %. The response shown in (b) is a typical fluorimeter recording. The units are units of fluorescence.
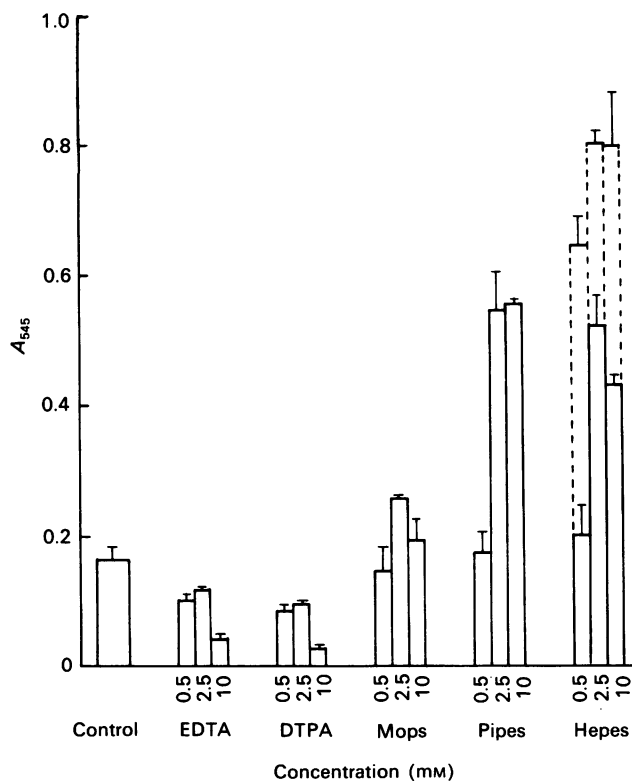

Fig. 4. Effects of various buffers and chelators at 0.5, 2.5 and 10 mM on deoxyribose (10 mM) degradation induced by 5 mM-H_2O_2 and 500 μM-$CuSO_4$ in 10 mM-sodium phosphate buffer, pH 7.2

Broken lines represent the response in the absence of phosphate buffer. The A_{545} values are given as the means ± S.D. for three determinations.

all three components were present, indicating a small but obviously important interaction between these components. However, these spectra were unstable and did not lend themselves to further analysis. In both cases the disappearance of the absorption peaks was accompanied by the appearance of a precipitate. Precipitate formation also occurred in the complete radical-generating system. Even if the precipitate was allowed to form this had no effect on the magnitude and kinetics of a subsequent CL response initiated by H_2O_2. In fact, if the supernatant above the precipitate was removed after centrifugation it elicited a CL response comparable with that of the system from which it was derived, indicating sufficient Cu^{2+} was still available in solution. This is in agreement with the dose-response study with Cu^{2+} in the complete radical-generating system, which indicates that less than 10 % of the Cu^{2+} normally added (500 μM) is actually required for an equivalent CL response. In contrast, the precipitates formed were inactive unless resuspended in a Hepes-containing medium, showing that Cu^{2+} needs to be dissolved to become reactive in our test reaction.

The effect of phosphate buffer on radical generation is complicated. In the deoxyribose degradation system the stimulation by Hepes was greater in the absence of phosphate buffer. The Hepes-dependent CL response requires a moderate concentration of phosphate buffer if CL is to be produced over a long period, but high concentrations of phosphate inhibit CL (Fig. 2). It is clear that the effect of phosphate is not, as might be expected, simply a concentration-dependent inhibition due to precipitation of copper phosphate, although this could well account for the inhibitory effect of high phosphate concentrations. The stimulatory effects of phosphate could possibly be due to multiple interactions of the system components facilitating the redox reaction

and/or an indirect one such as the ionic strength of phosphate increasing the sensitivity of the detection system, in this case CL.

Taken together, these data suggest that the role of Hepes is to keep Cu^{2+} as a soluble and active complex. The formation of an appropriate and soluble complex seems to be crucial since in the absence of phosphate and Hepes dissolved Cu^{2+} is inactive. It has previously been reported that the 'Good' buffers show negligible co-ordination with metal ions (Good et al., 1966), but this conclusion has been challenged in a paper reporting the formation of a complex between Pipes and Co^{2+} ions (Evans & Wood, 1987).

In summary we believe that Hepes, Pipes and histidine stimulate Cu^{2+}-dependent H_2O_2 breakdown by the formation of an active complex, whereas EDTA, DTPA and phosphate ions form inactive or poorly active complexes. This effect of Hepes is not seen with iron salts (results not shown).

Hepes and structurally related 'Good' buffers are widely used in biological research. In the light of our results, one should also be aware of the complexities of using Hepes and related buffers in the presence of Cu^{2+} ions and H_2O_2 or H_2O_2-generating systems. Another reason for avoiding Hepes is that Hepes/flavin mixtures (e.g. in media for cell cultures) can lead to H_2O_2 generation in the presence of light (Halliwell & Butt, 1972).

REFERENCES

Campbell, A. K., Hallet, M. & Weeks, I. (1985) Methods Biochem. Anal. **31**, 317–416

Clark, I. A., Cowden, W. B. & Hunt, N. H. (1985) Med. Res. Rev. **5**, 297–332

Dean, R. T. (1987) FEBS Lett. **220**, 278–282

Easmon, C. S. F., Cole, P. J., Williams, A. J. & Hastings, M. (1980) Immunology **41**, 67–74

Evans, D. F. & Wood, D. (1987) J. Chem. Soc. Dalton Trans. 3099–3101

Good, N. E., Winget, G. D., Winter, W., Connolly, T. M., Izawa, S. & Singh, M. M. (1966) Biochemistry **5**, 467–477

Graf, E., Mahoney, J. R., Bryant, R. G. & Eaton, J. W. (1984) J. Biol. Chem. **259**, 3620–3624

Grootveld, M. & Halliwell, B. (1986) Free Radical Res. Commun. **1**, 243–250

Gutteridge, J. M. C. (1987) Biochem. J. **243**, 709–714

Gutteridge, J. M. C. & Wilkins, S. (1983) Biochim. Biophys. Acta **759**, 38–41

Halliwell, B. & Butt, V. S. (1972) Biochem. J. **129**, 1157–1158

Halliwell, B. & Gutteridge, J. M. C. (1981) FEBS Lett. **128**, 347–352

Halliwell, B. & Gutteridge, J. M. C. (1984) Biochem. J. **219**, 1–14

Halliwell, B. & Gutteridge, J. M. C. (1986) Arch. Biochem. Biophys. **246**, 501–514

Hicks, M. & Gebicki, J. M. (1986) FEBS Lett. **199**, 92–94

Hunt, J. V., Simpson, J. A. & Dean, R. T. (1988) Biochem. J. **250**, 87–93

Johnson, G. R. A., Nazhat, N. B. & Saadalla-Nazhat, R. A. (1985) J. Chem. Soc. Chem. Commun. **7**, 407–408

Muller-Peddinghaus, R. (1984) Int. J. Immunopharmacol. **6**, 455–466

Sies, H. (ed.) (1985) Oxidative Stress, pp. 1–507, Academic Press, London

Slater, T. F. (1972) Free Radical Mechanisms in Tissue Injury, pp. 1–283, Pion, London

Slater, T. F. (1984) Biochem. J. **222**, 1–15