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THE BACTERICIDAL ACTION OF PEROXIDES; AN E.P.R. SPIN-TRAPPING STUDY

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E.P.R. spin trapping has been employed to study radical production during the bactericidal action of three peroxide compounds (peracetic acid, 4-percarboxy-N-isobutyltrimellitimide and magnesium monoperoxyphthalate) upon both Gram negative (*Escherichia Coli*) and Gram positive (*Staphylococcus Aureus*) bacteria. Use of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) has allowed direct detection of both carbon-centred and hydroxyl radicals, which are produced at varying rates for the different bacteria/peracid systems studied. The inhibition of bactericidal action, by DMPO and two antioxidants, Vitamin C and Trolox C, indicates that radicals are the lethal species and evidence is presented which suggests that radical production is internal to the bacterial cell. Hydroxyl radicals are believed to be the lethal species. The effect of added iron chelators and haem protein inhibitors indicates that iron species and haem proteins in particular are involved. A marked variation is found in observed hydroxyl-radical adduct signals with both the nature and concentration of peracid. A strong inverse correlation is found between the concentration of the observed radical adduct signal and the relative strength of the peroxide as a bactericide; use of a stable nitroxide as a radical scavenger confirms that strong bactericides produce radicals at a much faster rate than weak bactericides. Plots of radical generation versus time are correlated with % bacterial kill, offering further evidence that hydroxyl radicals are the lethal species.

KEY WORDS: Electron paramagnetic resonance, spin trapping, peroxygen, peroxide, peracid, *Escherichia Coli, Staphylococcus Aureus*, hydroxyl radicals, bactericides.

Abbreviations c.f.u./ml, colony-forming units per ml; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; *E. Coli, Escherichia Coli*; E.P.R., electron paramagnetic resonance; MMPP, magnesium monoperoxyphthalate; PAA, peracetic acid; PBTI, 4-percarboxy-N-isobutyltrimellitimide; *Staph. A., Staphylococcus Aureus.*

INTRODUCTION

Recent studies of the action of hydrogen peroxide upon bacteria suggest that the process of cell killing involves both intracellular iron and the production of hydroxyl radicals. Imlay *et al.*[1] have shown that *Escherichia Coli* (*E. Coli*) bacteria are protected from the lethal effects of hydrogen peroxide when the cells are pretreated with the iron chelators 1,10-phenanthroline or desferrioxamine (deferoxamine, Desferal®), potent iron(II) and iron(III) chelators respectively. Repine *et al.*[2] postulate the occurrence of an iron-catalysed Haber-Weiss cycle [reactions (1) and (2)], where the overall effect is shown in reaction (3). The addition of

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desferrioxamine prevents reduction of Fe^{3+} back to Fe^{2+} [reaction (1)] while 1,10-phenanthroline prevents oxidation of Fe^{2+} to Fe^{3+} [reaction (2)]. It has also been found that addition of the copper chelator neocupreine does not inhibit the lethal effect of hydrogen peroxide[1], suggesting that copper (which could feasibly replace iron in this cycle) does not play a major role in the bactericidal process.

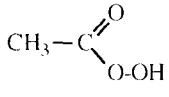
$$O_2^{-\cdot} + Fe^{3+} \longrightarrow O_2 + Fe^{2+} \quad (1)$$

$$H_2O_2 + Fe^{2+} \longrightarrow HO\cdot + HO^- + Fe^{3+} \quad (2)$$

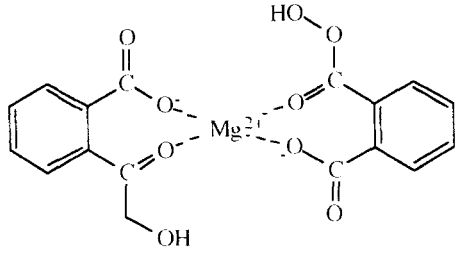
$$O_2^{-\cdot} + H_2O_2 \longrightarrow HO\cdot + HO^- + O_2 \quad (3)$$

The addition of a variety of hydroxyl radical scavengers has been shown to inhibit the bactericidal action of hydrogen peroxide upon *Staphylococcus Aureus* (*Staph. A.*), and it has also been found[2] that bacteria pre-incubated in broth media with increasing concentrations of $FeSO_4$ exhibit progressively enhanced susceptibility to killing by hydrogen peroxide. Addition of extrinsic iron(II) during bactericidal assay to bacteria, grown in media with no added iron, shows no similar enhancement of susceptibility to kill.[2]

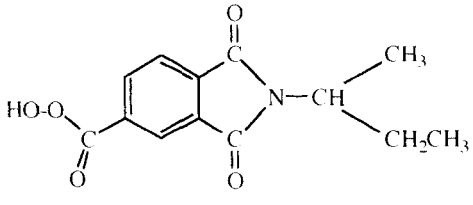
In this study we have used the technique of electron paramagnetic resonance (E.P.R.) spectroscopy together with spin-trapping techniques in order to obtain information on radical production by both Gram negative (*E. Coli*) and Gram positive (*Staph. A.*) bacteria upon treatment with the peroxides peracetic acid (PAA), magnesium monoperoxyphthalate (MMPP) and 4-percarboxy-N-isobutyl-trimellitimide (PBTI);



Peracetic acid, PAA

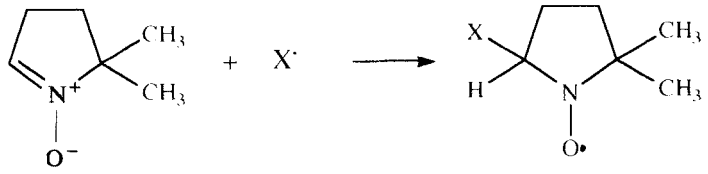


Magnesium monoperoxyphthalate, MMPP



4-Percarboxy-N-isobutyltrimellitimide, PBTI

Direct evidence for radical production has been obtained by the detection (by E.P.R.) of spin adducts generated via the reaction of the initially produced radical (X·) with the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), [reaction (4)]. Analysis of the E.P.R. spectra of the resulting nitroxide radicals, if formed, should allow information to be obtained as to the identity of X· and its rate of generation.



(4)

5,5-Dimethyl-l-pyrroline *N*-oxide

DMPO

Spin Adduct

The aims of this study are as follows: to confirm that radicals are the lethal species rather than artefacts of some other process; to identify the radical species generated; to discover whether radical generation is internal or external to the bacterial cell; to determine the role of transition metals in the process (and the effect of modulators of transition metal reactivity) in an attempt to deduce the mechanism of radical production; to determine the link between rate and extent of radical production and bactericidal activity; and to attempt to correlate radical generation and cell kill.

MATERIALS AND METHODS

Materials

Peracetic acid (PAA), magnesium monoperoxyphthalate (MMPP) and Trolox C® were obtained from Aldrich. 4-Percarboxy-N-isobutyltrimellitimide was supplied by Interox Services plc. 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was obtained from Aldrich, and purified by stirring a solution (0.22 M) of the spin trap in de-ionised water with a little activated charcoal for ca. 1 hour. After filtration, batches of this solution were frozen and stored in the dark until required. Desferrioxamine (Desferal®, deferoxamine mesylate) was obtained from Sigma. Sodium cyanide and sodium azide were purified by passing a solution of the appropriate dilution down a column of Chelex® 100 chelating resin (Bio-Rad) to remove any transition metal ions present. All other chemicals employed were commercial samples and used without further purification.

E. Coli (Strain NCIMB 9517) and *Staph. A.* (Strain NCIMB 9518) were obtained as freeze-dried bacterial cultures from The National Collections of Industrial and Marine Bacteria Ltd., and were grown at 37° C in 100 ml of nutrient broth medium (Oxoid® nutrient broth) prepared according to the manufacturer's instructions. After incubation for 24 hours, the bacteria were spun down, washed twice with and then finally resuspended in sodium sulphate solution (0.05 M). The bacterial content of a diluted aliquot of the suspension was determined spectrophotometrically, and the sample solution prepared by appropriate dilution to a bacterial suspension of 5.0×10^9 colony-forming units/ml (c.f.u./ml).

E.P.R. Spin-trapping Experiments

E.P.R. spectra were recorded at room temperature on a Bruker ESP-300 spectrometer equipped with an X-band klystron and 100 kHz modulation. The standard incubation consisted of the following final concentrations; bacteria (2×10^9 c.f.u./ml), DMPO solution (66 mM), peracid (0–0.36 mM), each sample made up to a total volume of 0.5 ml with de-ionised water. The sample was pipetted into an E.P.R. flat cell and scanning of the E.P.R. spectrum was begun 70 s after mixing, with a sweep time of 300 s (and repeated as often as necessary). Peak heights (which are directly proportional to the radical concentration) were measured on the lower field absorption line of the central pair of the lines of the 1:2:2:1 quartet of the hydroxyl radical adduct (see later), and are expressed in arbitrary units.

Study of the effect of metal ion chelators upon radical generation employed the same standard incubation system as detailed above, but the sample was made up to the required volume with an aqueous solution of chelating agent (at the appropriate concentration) prior to addition of peracid. A similar procedure for experiments to determine the effect of extrinsic iron upon radical generation was followed as for those on the effects of chelating agents; solutions of $FeSO_4$ or $FeCl_3$ at the appropriate concentrations replaced those of the chelating agents in the above.

Determination of the relative rates of radical generation by PAA and MMPP (both 0.06 mM) using di-t-butyl nitroxide employed the following final concentrations in the reaction system; *E. Coli* (2×10^9 c.f.u/ml), di-t-butyl nitroxide (0.1 mM), PAA or MMPP (0.06 mM) in 0.5 ml of sample. Scanning of the E.P.R spectrum was begun 70 s after mixing, with a 300 s sweep time (and repeated as often as necessary). Spin-trapping experiments, where attempts were made to correlate radical adduct generation with bacterial kill, followed the same procedure as the standard spin-trapping experiments outlined above, except that the appropriate signal was scanned at 60 s intervals.

Bacterial Experiments

Tests for inhibition of kill were performed as follows: 0.15 ml of inhibitor (at the appropriate concentration) was added to 0.2 ml of bacterial suspension (final concentration 2×10^9 c.f.u./ml), then 0.15 ml of peracid solution (final concentration 0.36 mM) was added. After the stated contact time, 1 ml of sodium thiosulphate (0.05 g/ml) was added to neutralise the peracid present. After 1 min., 0.1 ml of this reaction solution was added to 5 ml of sodium sulphate (0.05 M) and this process repeated for four successive dilutions. Finally, 0.1 ml of each diluted sample was taken, separately plated out with plate count agar (Oxoid®), incubated at 37° C for 48 hours, then the number of colonies counted. Controls carried out in the absence of both peracid and peracid and thiosulphate indicated that the neutralising agent did not affect the viability of the bacteria at the concentration employed. For studies of the effect of intrinsic iron upon the rate of radical generation, increasing concentrations (200–800 μM) of $FeSO_4$ or $FeCl_3$ were added to the nutrient broth medium prior to innoculation with bacteria.

Plots of bacterial kill versus time were performed according to the following procedure: a solution of the peracid at twice the desired concentration was prepared in deionised water within 60 mins. of the start of the test. A 5 ml portion of the peracid was placed in a sterile test tube. At $t = 0$, 4 ml of deionised water was added

and mixed, followed at $t = 30$ s by 1 ml of a microbial suspension (final concentration in solution 2×10^9 c.f.u./ml). At $t = 330$ s, a 1 ml sample was added to 9 ml of neutraliser solution (made by dissolving 0.25 g catalase (Sigma C-10) in 1 l of 50 g/l sodium thiosulphate). At $t = 630$ s, two 1 ml samples were plated out and incubated as described above. Colonies were counted and % bacterial kill values calculated with reference to experiments where the same procedure had been followed, except that 5 ml of water replaced the 5 ml of peracid in the initial step.

In all cases, except where indicated otherwise, the reported data are from single experiments which were typical of several separate determinations.

RESULTS AND DISCUSSION

Since it was expected that the radicals produced (for example the hydroxyl radical) would be short-lived or produced at a slow rate, and hence would not reach high enough steady state concentrations for direct detection, we employed the spin-trapping method.[3] In this technique, the radicals react with an added spin trap (usually a nitroso or nitrone compound) to produce a longer-lived free radical which can be conveniently detected, and often unambiguously identified, by E.P.R. spectroscopy. We have used the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), which reacts with hydroxyl radicals to give a characteristic 1:2:2:1 quartet ($a_N = a_{\beta-H} = 1.49$ mT) for the hydroxyl radical adduct.[4]

Initial Spin-trapping Experiments

In initial spin-trapping experiments, it was found that signals could be observed by E.P.R. spectroscopy for a variety of peracids over a range of concentrations (typically ≤ 0.36 mM) in the presence of DMPO with both *E. Coli* and *Staph. A*.

Thus, upon reaction of peracetic acid (PAA, 0.015–0.12 mM), magnesium mono peroxyphthalate (MMPP, 0.6–0.36 mM) or 4-percarboxy-N-isobutyltrimellitimide (PBTI, 0.03–0.36 mM) with either *E. Coli* or *Staph. A*. in the standard trapping experiments (see Materials and Methods), signals which can be assigned to the hydroxyl radical adduct were observed, Figure 1. No such signals were observed in the absence of either bacteria, spin trap or peracid. The intensity of the observed hydroxyl radical signals increased with higher concentrations of spin trap (up to 0.13 mM) or bacteria (up to 5×10^9 c.f.u./ml). At low concentrations (< 0.06 mM) of PBTI with *Staph. A*., additional adduct signals were observed (with $a_N = 1.58$ mT, $a_{\beta-H} = 2.54$ mT) consistent with the production of carbon-centred radicals. The hydroxyl radical adduct signals were found to increase with time after mixing until a point at which a maximum adduct signal was observed. As can be seen in Figure 2, in which observed peak height is plotted against incubation time for MMPP with both *E. Coli* and *Staph. A*., it was generally found that the time to reach this maximum signal varied markedly with peracid concentration.

It was also noted that plots of the relative radical concentration versus time showed marked differences in behaviour for the three peroxides (Figure 3). Reasons for the variations illustrated in Figures 2 and 3 are proposed below.

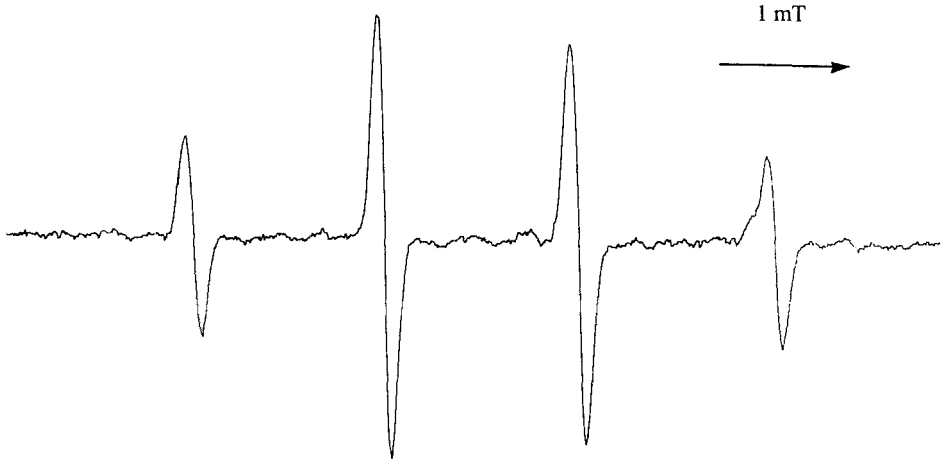
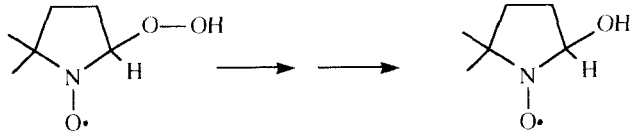


FIGURE 1 E.P.R. spectrum of the hydroxyl-radical adduct of DMPO, generated by reaction of MMPP (0.06 mM) with *Staph. A.* (2×10^9 c.f.u./ml) in the presence of DMPO (66 mM).

Effect of Radical Scavengers upon Bactericidal Action

It has been shown[2] that a variety of hydroxyl radical scavengers, including thiourea and mannitol, produce marked inhibition of the lethal effects of hydrogen peroxide upon *Staph. A*. This suggests that hydroxyl radicals are the killing agents rather than artefacts of some other process. However, it is possible that the ·OH-adducts observed by spin-trapping experiments reported herein are produced by some other reaction, such as the decomposition of the superoxide (O_2^{-}·) radical adduct[5] [reaction (5)] or hydration of the trap after it has been subjected to oxidation[6] [reactions (6) and (7)];



(5)

$$DMPO + X^{+} \dashrightarrow DMPO^{+\cdot} + X \quad (6)$$

$$DMPO^{+\cdot} + H_2O \dashrightarrow \textbf{DMPO-OH}^{\cdot} + H^{+} \quad (7)$$

In order to determine whether the observed signals are indeed due to trapping of hydroxyl radicals produced during the bactericidal process, a series of experiments has been performed to discover whether the presence of DMPO had an inhibiting effect upon the killing action of the peroxides. Table 1 shows the effects of DMPO and two common biological antioxidants, vitamin C and Trolox C (a water-soluble analogue of vitamin E), upon the bactericidal action of PAA with *Staph. A.*, expressed as a percentage of a control with no inhibitor added.

The inhibiting actions of DMPO, vitamin C and Trolox C strongly suggest that

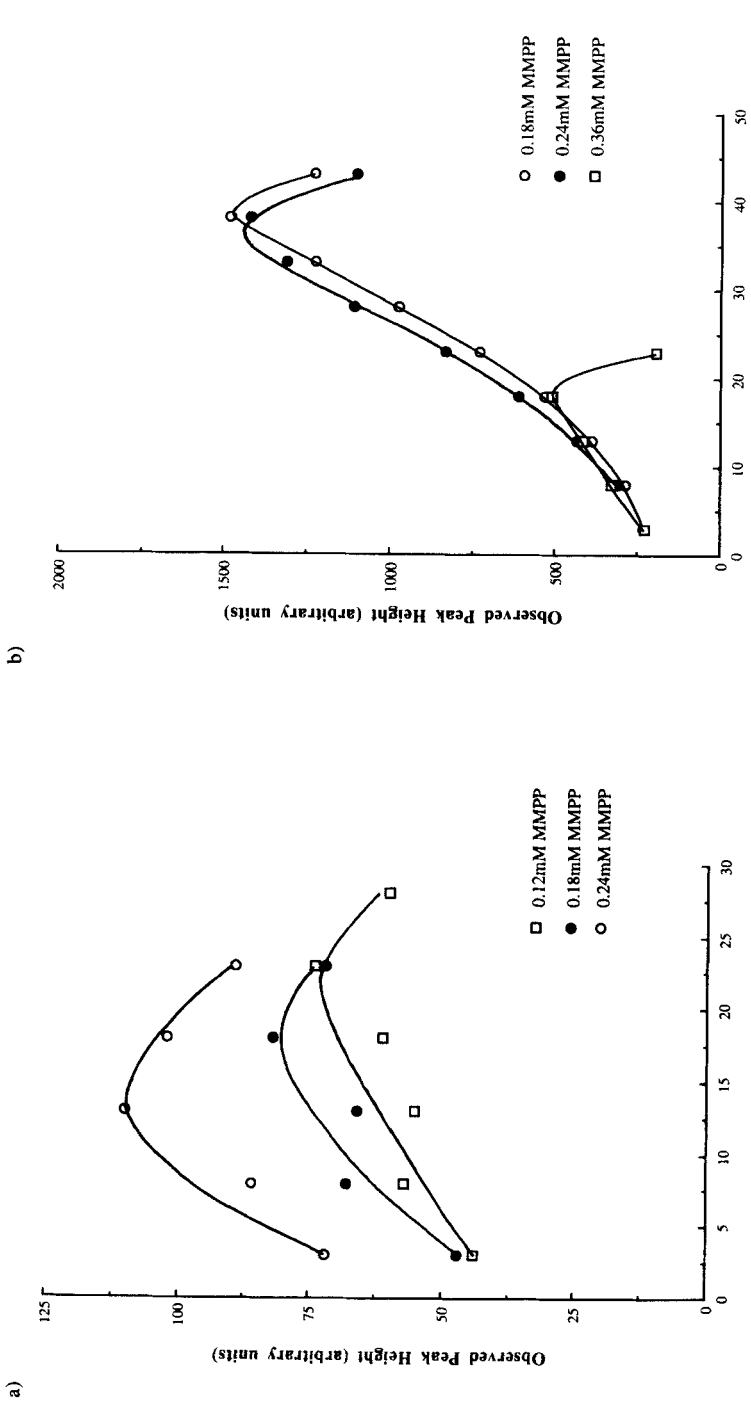


FIGURE 2a,b Plot of the intensity of the hydroxyl-radical adduct signal versus time for varying concentrations of MMPP in the presence of DMPO (66 mM) with a) *E. Coli* (2×10^9 c.f.u./ml); b) *Staph. A.* (2×10^9 c.f.u./ml).

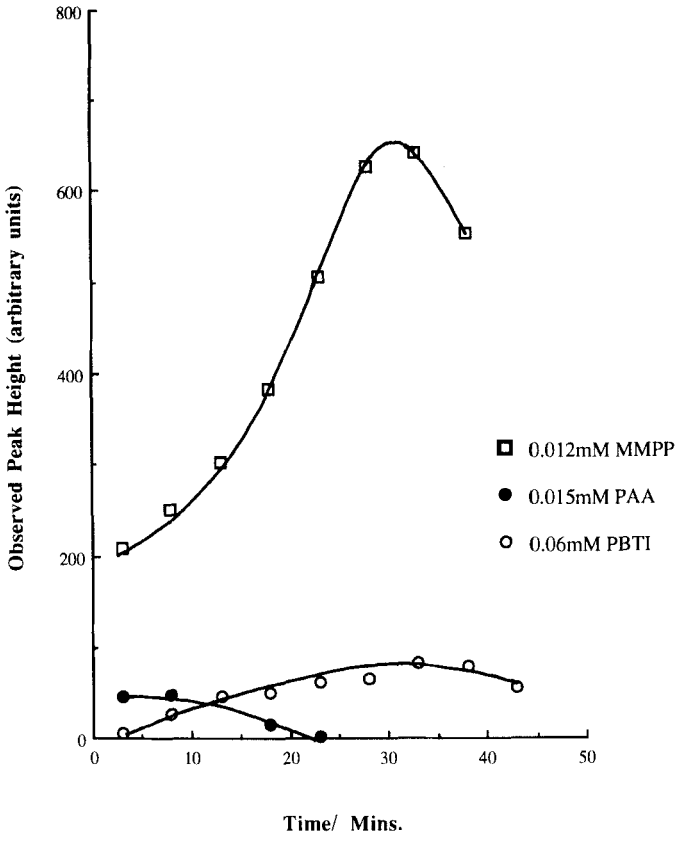


FIGURE 3 Plot of the intensity of the hydroxyl-radical adduct signal versus time for *Staph. A.* (2×10^9 c.f.u./ml) for peroxides studied, at varying concentrations, in the presence of DMPO (66 mM).

TABLE 1
Effect of inhibitors upon killing of *Staph. A.* by peracetic acid

Inhibitor added	Time of exposure to PAA	% Inhibition of bactericidal activity
DMPO (66 mM)	3 mins.	66 ± 2.6 (5)[a]
DMPO (66 mM)	6 mins.	55 ± 4.2 (5)
Vitamin C (5 mM)	5 mins.	68 ± 7.6 (3)
Trolox C (5 mM)	5 mins.	43 ± 9.2 (3)

[a] Mean ± standard deviation (number of determinations) from samples of *Staph. A.* (2×10^9 cfu/ml) treated with PAA (final concentration 0.36 mM), see Materials and Methods. Controls indicated that these inhibitors alone did not affect viability of bacteria at the concentrations employed here.

radicals are involved in the bactericidal action. The fact that the bactericidal action *is* inhibited by Trolox C, vitamin C and DMPO (which have very slow rates of reaction with $O_2^{-\cdot}$ and $HOO\cdot$)[7-9] suggests that neither $O_2^{-\cdot}$ nor $HOO\cdot$ is responsible for the observed bactericidal action. This suggests that hydroxyl radicals are the lethal species and that the observed radical adducts are arising from the direct trapping of hydroxyl radicals.

Effect of Addition of a Membrane-Impermeable Line Broadening Agent upon Observed Radical Adduct Signals

Though radical adduct signals arising from the trapping of $\cdot OH$ are observed, this gives no indication as to whether radical production is internal or external to the cell as both the spin trap DMPO and the resultant spin adducts are known to be freely membrane permeable.[10] In order to determine whether the observed radical production occurs inside or outside the bacterial cell, we employed the membrane-impermeable agent potassium ferricyanide.[11] This compound broadens the E.P.R. signals of radicals it encounters by paramagnetic broadening. The fact that it is membrane-impermeable would lead us to expect that it would only broaden radicals present external to the bacterial cell. Thus should radical production be occurring inside the cell, it was expected that addition of ferricyanide would have no effect upon the observed radical adduct signals.

In initial experiments with *Staph. A.* (2×10^9 c.f.u./ml) and MMPP (0.12 mM) signals could not be observed in the presence of ferricyanide (10 mM); this may be due to paramagnetic broadening. However, it was found that at much lower concentrations of MMPP (< 0.012 mM), weak but well-defined $\cdot OH$-adduct signals were observed; these signals disappeared after a short period of time. These observations are interpreted as being due to radical production (and subsequent trapping) *within* the cell; the subsequent loss of the signals is believed to be due to gradual disruption of the membrane (rendering it permeable to ferricyanide and resulting in the loss of the signals due to paramagnetic broadening) as a result of the bactericidal activity of MMPP. Disruption of the bacterial membrane by these low concentrations of MMPP has been confirmed by measurement of enzyme leakage.[12]

Effect of Transition Metal Chelators and Metallo-Protein Inhibitors upon Observed Radical Adduct Generation

A series of spin-trapping experiments was carried out in the presence of a variety of common transition-metal chelating agents and metallo-protein inhibitors (Imlay and co-workers showed that both desferrioxamine and 1,10-phenanthroline protected *E. Coli* from the lethal effects of hydrogen peroxide[1]). The behaviour of the standard spin-trapping systems for *Staph. A.* (2×10^9 c.f.u./ml) with PAA (0.03 mM) and MMPP (0.06 mM) was studied in the presence of such agents, and comparison made with signals observed in their absence. Results are shown in Table 2.

For both a strong bactericide such as PAA and a weak bactericide such as MMPP, the addition of the potent iron(III)-chelator desferrioxamine ($>300\,\mu$M) to the trapping system led to the loss of the $\cdot OH$-adduct signals. This offers strong evidence that iron species [particularly iron(III)] are involved in the radical generating process. In contrast, the addition of 200 μM 1,10-phenanthroline [an

TABLE 2
Effect of transition metal chelators and metallo-protein inhibitors upon intensity of hydroxyl radical adduct for *Staph. A.*[a]

Chelating Agent	PAA (0.03 mm)	MMPP (0.06 mM)
Desferrioxamine (>300 μM)	No signals observed	No signals observed
1,10-phenanthroline (200 μM)	15% enhancement	30% reduction
1,10-phenanthroline (500 μM)	30% reduction	50% reduction
Cyanide (1 mM)	----------	75% reduction
Azide (1 mM)	----------	60% reduction

[a] Bacterial concentration 2×10^9 c.f.u./ml; for detailed conditions see Materials and Methods.

iron(II) and copper(II) chelating agent] produced a different effect for each of the peracids studied. For PAA, a small but well-defined increase in signals was observed, while for MMPP, the opposite effect was noted; the intensity of the ·OH-adduct signals were very much less than those observed in the absence of 1,10-phenanthroline. At 500 μM 1,10-phenanthroline, however, both peroxides produced less intense ·OH-adduct signals than in the absence of the chelator. Reasons for these observed differences are proposed below.

Efforts were also made to block metallo-proteins [e.g. iron(III) haem proteins] within the bacterial cell. Both cyanide and azide are commonly-used inhibitors of such species, and it was hoped that addition of these would provide more detailed information upon the possible role of specific metallo-proteins in the process of radical generation. Problems were encountered with the presence of adventitious transition-metal ions in the samples of sodium cyanide and sodium azide used; despite exhaustive efforts to remove such impurities with chelating resin (see Materials and Methods), it was found that, even in the absence of bacteria, PAA (0.03 mM) produced large quantities of hydroxyl radicals in the presence of these chelators, presumably by transition metal ion catalysed decomposition. Similar observations were made with PBTI (0.06 mM). Thus it was found that very large background ·OH-adduct signals had to be taken into consideration; this rendered determination of any relative changes in the characteristically small ·OH-adduct signals very difficult.

However such background ·OH-adduct signals observed for MMPP (0.06 mM) with both cyanide and azide were not only very much smaller, but the characteristically large signals observed in the absence of such chelators meant that subtraction of such blanks from signals observed in the presence of cyanide or azide produced results that were much more reliable than was the case with PAA or PBTI. On addition of cyanide (1 mM) or azide (1 mM) prior to MMPP (0.06 mM) to *Staph. A.* (2×10^9 c.f.u./ml), it was found that the observed ·OH-adduct signals were considerably less intense than those observed in the absence of the chelating agents. This suggests that haem proteins may be at least involved in hydroxyl-radical generation; however the inhibiting effect noted for 1,10-phenanthroline – an iron(II) chelator which would leave the iron(III) sites in the haem proteins essentially unaffected – in the same system indicates that these may not be the sole sites of radical generation.

Effect of Additional Intrinsic and Extrinsic Iron upon Observed Radical Adduct Generation

In order to study the role of iron in radical generation, a series of E.P.R. experiments were performed to determine the effect of iron added to the bacteria upon the observed ·OH-adduct signals. A series of spin-trapping experiments were performed using *Staph. A.* (2×10^9 c.f.u./ml) which had been grown in broth media containing increasing concentrations of iron(II) (in the form of $FeSO_4$) or iron(III) (as $FeCl_3$). These were spun down and treated in the standard spin-trapping system with MMPP (0.12 mM) or PBTI (0.06 mM). In Figures 4a,b, comparison is made with the radical adduct signal observed for *Staph. A.* grown in standard broth media with the appropriate peroxide compound.

It can be seen that with MMPP (0.12 mM), addition of both iron(II) and (III) to the growth medium prior to innoculation with bacteria serves to enhance the ·OH-adduct signal seen in the absence of added transition metal by as much as 300%. This observation offers further evidence of the increased susceptibility of such iron-rich bacteria to the lethal effects of hydrogen peroxide, as shown by Repine and co-workers.[2] However, at very high concentrations of metal ions, this enhancement tails off: in the case of iron(II), at a concentration of 1 mM, to such an extent that the observed signal drops below that expected for *Staph. A.* grown in the standard media. With PBTI (0.06 mM), no such initial signal enhancement is observed; in fact a steady reduction in adduct signal is observed across the range of iron(II) concentrations studied (0–1 mM), while for iron(III) an initial reduction in observed signal intensity with increasing metal ion concentration is followed by a large increase in signals at elevated iron(III) levels. Possible reasons for these differences in behaviour are proposed below. In all cases, the incubation times required to reach the maximum observed radical adduct signal decreases with increasing concentration of added iron, suggesting that radicals are being generated at increased rates in the presence of higher concentrations of iron (Figure 5).

The process by which such additional iron is stored is unclear. Gutteridge *et al.*[13] noted that *Staph. A.* grown in media with increasing concentrations of $FeSO_4$ appeared to accumulate this iron in a predominantly non-protein-bound form (presumably as a result of saturation of iron storage proteins). It is possible that the observed increase in radical production at elevated levels of additional iron is due to reaction between the peracid and this "free" iron.

It has also been noted that iron(II) added to pre-grown bacteria during the bactericidal assay did not appreciably increase killing of *Staph. A.* by hydrogen peroxide.[2] E.P.R. spin-trapping studies (over a similar peracid concentration range) of the effect of adding increasing concentrations of extrinsic iron(II) (200–800 μM) or iron(III) (200–800 μM) to both *E. Coli* and *Staph. A.* immediately prior to addition of PAA, MMPP or PBTI (0.06 mM) indicate that in all cases very large initial bursts of hydroxyl radicals are observed. The resultant radical adduct signals disappeared within a matter of minutes, most probably due to extensive radical-radical reactions. The observation that these have little lethal effect upon the bacteria would seem to suggest that the majority of these reactions are taking place in the bulk solution (and hence external to the bacterial cell) rather than internal to the bacteria, which our studies suggest to be a prerequisite for lethal damage to occur.

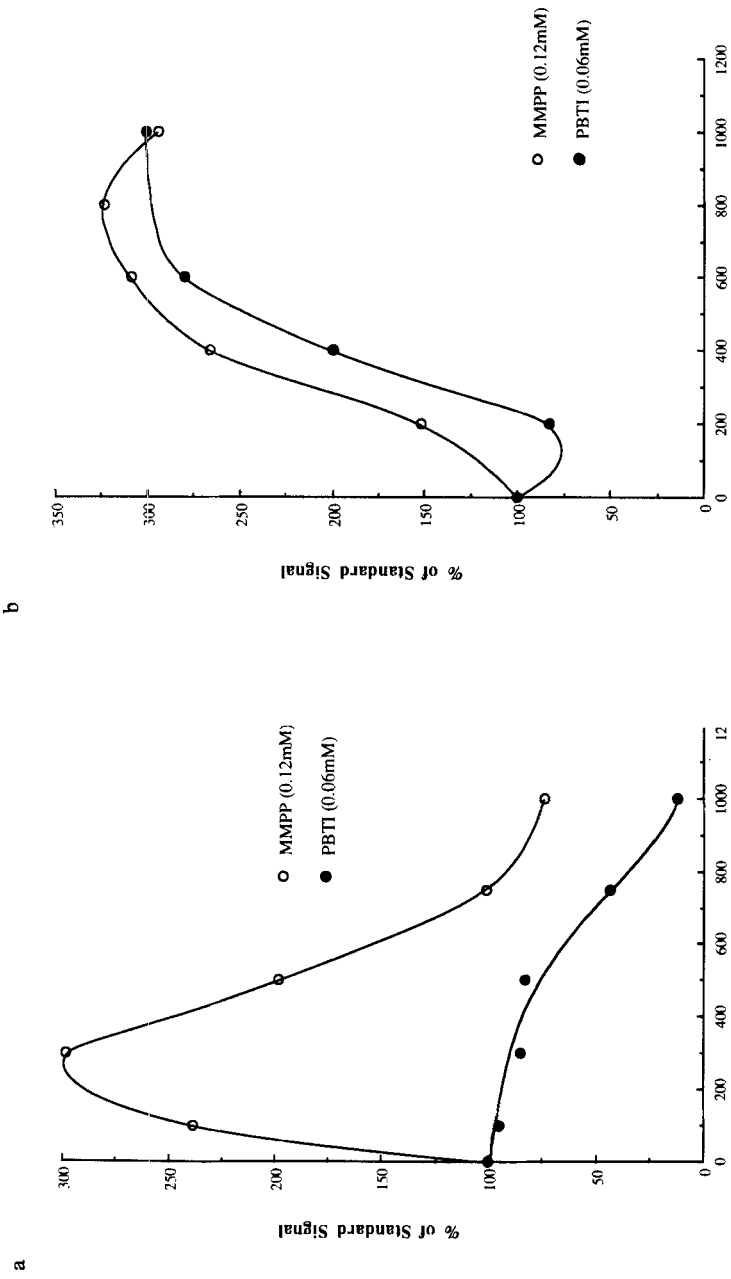


FIGURE 4a,b Comparison of the effects of addition of varying concentrations of a) Fe(II) (as $FeSO_4$); b) Fe(III) (as $FeCl_3$) to the growth medium in spin-trapping experiments where PBTI (0.06 mM) or MMPP (0.12 mM) was incubated with *Staph. A.* (2×10^9 c.f.u./ml) in the presence of DMPO (66 mM). The signal intensity of the hydroxyl-radical adduct is expressed as a percentage of the signal intensity determined in the absence of added a) Fe(II); b) Fe(III).

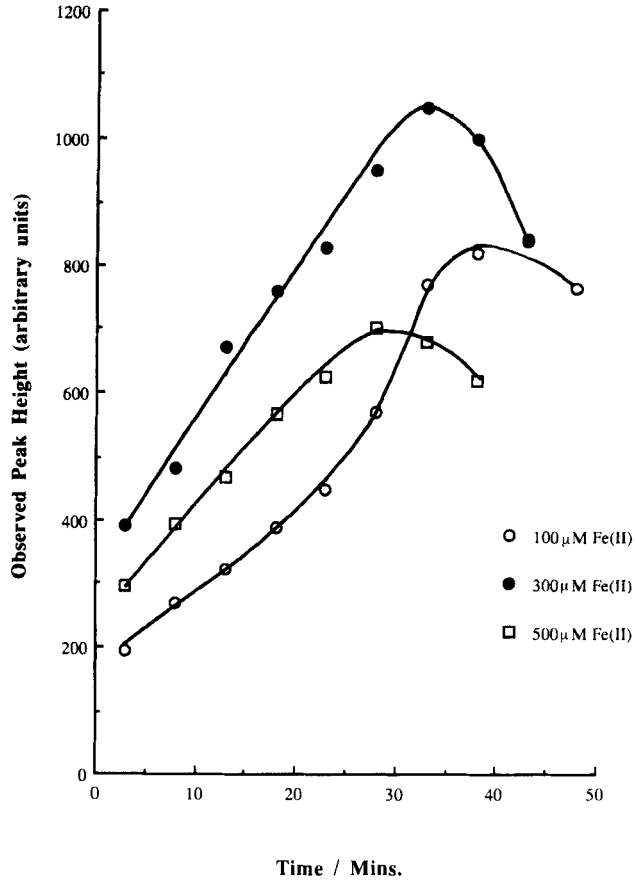


FIGURE 5 Plots of the intensity of the hydroxyl-radical adduct signal versus time for experiments where *Staph. A.* [$(2 \times 10^9$ c.f.u./ml), bacteria grown in varying concentrations of Fe(II)] were incubated with MMPP (0.12 mM) in the presence of DMPO (66 mM).

Radical Generation Profiles

The observed maximum ·OH-adduct signal was found to vary widely with concentration for all the peracid/bacteria trapping systems studied. In order to determine whether there was a possible relationship between such behaviour and bactericidal activity, radical generation profiles were plotted for each system (final bacterial concentration 2×10^9 c.f.u./ml) across a concentration range of 0–0.36 mM peracid (Figure 6). In these plots the maximum observed peak height (i.e. radical adduct concentration) over the period of study is plotted against peracid concentration for both bacteria.

Several features are apparent from comparison of these plots. In all cases, the observed radical-adduct concentration increases with peracid concentration, until the observed signals reach a maximum for the system. As the peracid concentration is increased beyond this value, the observed adduct signals progressively decrease. In the case of PBTI and *Staph. A.*, Figure 6b, a secondary maximum is found at

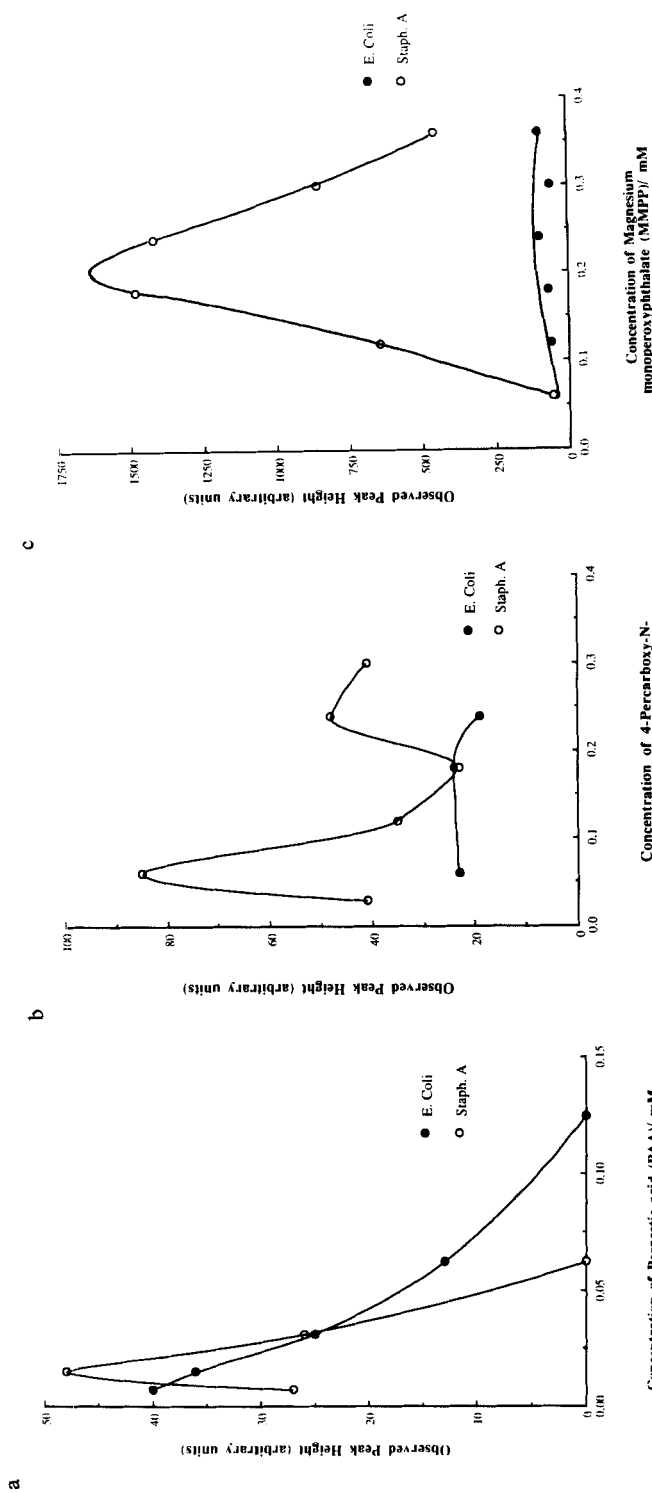


FIGURE 6a,b,c Plots of the maximum hydroxyl-radical adduct signal versus concentration for *E. Coli* (2×10^9 c.f.u./ml) or *Staph. A.* (2×10^9 c.f.u./ml), in the presence of DMPO (66 mM) with varying concentrations of a) PAA; b) PBTI; c) MMPP.

very high peracid concentrations. For all the peracids studied, the adduct signals (i.e. radical concentrations) observed for *Staph. A.* are appreciably larger than those found with *E. Coli.* This suggests that membrane penetrability may be an important factor in the bactericidal ability of a given peracid, but could equally be due to differences in composition with respect to metal ions and proteins, defensive systems (enzymes, antioxidants) or susceptibility of vital targets between the two bacteria.

If killing does follow a radical mechanism, as suggested by the above data, it might be expected that the nature of the radical generation profiles would vary according to the bactericidal efficiency of the peracid. Thus for a "good" bactericide, increasing the concentration of peracid from a low value would produce a rapid increase in radical generation and hence a marked build-up in the the observed maximum radical adduct signal until some point – the "optimum concentration" – after which radical-radical termination become significant and the overall intensity of the observed adduct signals tail off. For a "poor" bactericide, the initial increase in radical production with increasing peracid concentration would be expected to be less marked, and hence the optimum concentration of peracid to be much higher than for the strong bactericide.

From Figure 6 it can be seen that with *Staph. A.*, PAA, PBTI and MMPP show optimum concentrations, for the system under study, of ca. 0.02, ca. 0.075 and ca. 0.2 mM respectively, while for *E. Coli* the values are <0.01, ca. 0.125 and >0.3 mM. Bactericidal assays with these three peroxides indicate that PAA and PBTI are very strong bactericides, while MMPP is very weak.[14]

Hence, particularly in the case of *Staph. A.*, the observations at least partially support the hypothesis that a *low* optimum concentration of peracid (as judged by these spin-trapping experiments) is indicative of a *strong* bactericide, and a *high* value suggests *weak* bactericidal action. Indeed, in preliminary experiments employing twelve other peroxide compounds, including aliphatic and aromatic species, there appears to be an excellent correlation between low optimum concentration and subsequent good performance in bactericidal assays.[15]

A further feature noted across the range of peracids studied was a strong inverse correlation between the size of maximum radical-adduct signal observed at the optimum peracid concentration and bactericidal ability of the peracid. This further supports the hypothesis that strong bactericides (such as PAA; maximum peak height for *E. Coli* 40, *Staph. A.* 48) appear to produce an initial large burst of radicals, only a proportion being detected by E.P.R. as the corresponding spin-adducts, the others being lost through radical-radical and radical-molecule reactions. Conversely, weak bactericides (such as MMPP, maximum peak height for *E. Coli* 100, *Staph. A.* 1620) seem to produce radicals at a much slower rate, the resultant adduct signal gradually increasing, in the case of *Staph. A.* and MMPP (0.18 mM) over a period of up to 50 mins. The lack of killing (i.e. inactivation of the radical generation system) is probably the more important factor – the overall amount of radicals generated with MMPP may be equal (or even greater) than with PAA and PBTI, but they are generated over a longer time period and hence result in a much slower build up of the (ultimately much larger) hydroxyl-radical adduct signal. Experiments designed to test this hypothesis are described below.

The reasons for the secondary maximum observed with *Staph. A.* and PBTI in Figure 6b (and indeed with *Staph. A.* and a series of N-alkyl substituted 4-percarboxytrimellitimides) are unclear. It is likely that the subsequent increase in radical production is due to cell lysis or decompartmentalisation of components

(e.g. transition metals) of the bacterial cell during necrosis, and that these components subsequently react with the peracid to produce hydroxyl radicals.

The previously noted differences in the observed effects upon hydroxyl radical generation of addition of 200 μM 1,10-phenanthroline to *Staph. A.* (2×10^9 c.f.u./ml) between PAA and MMPP (Table 2) could be explained with reference to the relevant points on Figure 6. For PAA (0.03 mM, Figure 6a) tail-off of the observed radical adduct signals is already occurring, presumably due to high levels of radical-radical reactions. The observation that 1,10-phenanthroline protects *E. Coli* from the lethal effects of hydrogen peroxide[1] might suggest that if it offers the same protection to *Staph. A.* in the presence of PAA, then it would serve to slow down radical production. This would imply that the extent of radical-radical reactions occuring in the rapid initial burst of radicals found in the absence of such protection would likewise decrease and hence, as is the case in this experiment, observed radical adduct signals would increase. However at higher concentrations of 1,10-phenanthroline (500 μM), protection, and hence inhibition of radical generation, is such that smaller adduct signals are observed.

In contrast MMPP (0.06 mM, Figure 6c), even in the absence of such protection, is presumably producing radicals slowly enough that the reduction in the rate of radical generation produced by the presence of 1,10-phenanthroline is merely observed as a reduction in observed ·OH-adduct signals.

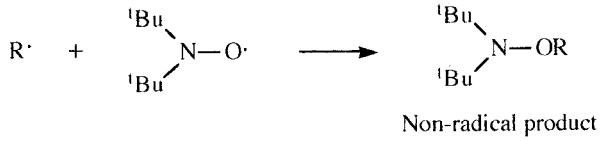
Comparison with the corresponding points for MMPP (0.12 mM) and PBTI (0.06 mM) with *Staph. A.* on Figures 4 and 5 suggests that the effect of increasing the iron concentration in the growth medium prior to innoculation with bacteria is to simulate the radical generation that would be otherwise observed at higher concentrations of the relevant peracid. Further evidence for increasing rates of radical production with higher concentrations of both iron(II) and iron(III) is offered by the observation that incubation times required to attain maximum observed radical adduct signals decrease progressively with increasing concentration of additional iron. Thus for weak bactericides such as MMPP, the presence of low concentrations of either additional iron(II) or iron(III) in the growth medium stimulates radical production such that the observed ·OH-adduct signals progressively increase; at high concentrations of additional iron the observed signals tail off due to radical-radical reactions.

For strong bactericides such as PBTI, such tail-off of observed radical adduct signals is already apparent at a concentration of peracid of 0.06 mM with normally-grown *Staph. A.*, and additional iron(II) merely serves to stimulate radical production such that observed signals decrease further in size. This would suggest that radical generation is already extremely rapid and extensive at this concentration of PBTI and that the bacteria are already dying very quickly; additional iron(II) does not give any marked in the rate or extent of radical production over and above that which is already occurring.

The initial effect of additional iron(III) was to produce a similar reduction in signals. However, radical production at higher concentrations of transition metal is enhanced to such an extent that the secondary maximum (previously discussed for Figure 6b) in radical production is observed, presumably due to lysis and decompartmentalisation as discussed earlier.

Study of the Rate of Radical Generation

The proposal that rates of radical generation vary according to bactericidal ability was tested by observation of the effect of the *E. Coli*/peracid system (in the absence of spin trap) upon a standard nitroxide radical, di-t-butyl nitroxide. It was hoped that this compound would enable us to monitor the relative rates of radical production *in situ*, by means of its action as a radical scavenger. Addition of this compound to the bacterial sample resulted in the detection of a 1:1:1 triplet E.P.R. signal characteristic of a free-tumbling nitroxide species. Subsequent addition of peracid to the sample led to a reduction in the observed signal with time, (presumably as radicals produced by the bacteria/peracid system reacted with the nitroxide species), although this might not only be due to the desired reaction [reaction (8)], but also due to molecular oxidation of the nitroxide by the peracid alone. The latter pathway has been shown to be unimportant in control experiments which indicated that there was negligible reaction between di-t-butyl nitroxide and either PAA or MMPP in the absence of bacteria.



(8)

Non-radical product

When di-t-butyl nitroxide (0.1 mM) was incubated with *E. Coli* (2×10^9 c.f.u./ml), and peracid (0.06 mM) added, the reduction of the nitroxide signal was very fast for PAA, indicating rapid radical generation, but very slow for MMPP, suggesting much slower radical production (Figure 7). These observations confirm that bactericidal activity can be directly correlated with radical-generating ability in the peroxide/bacteria system.

Correlation between Radical Production and Bacterial Kill

Although attempts to correlate observed ·OH-adduct generation and bacterial kill were made (Figure 8), it was in the knowledge that the protection from bactericidal action provided by the spin trap present necessitated that only an approximate correlation could be hoped for. The % bacterial kill versus time plots were produced with the same concentrations of peracid and bacterial cell numbers as the experiments for the radical generation plots but in the absence of spin trap (which would be expected to increase bactericidal activity; see above). Hence the % bacterial kill that is occurring in the former plots is probably a good deal higher than that which is taking place in the corresponding radical-adduct generation plots (where the spin trap must be present). Figure 8 show plots of % bacterial kill versus time, alongside observed peak height of ·OH-adduct (expressed as a % of the largest adduct signal observed for the peracid/bacteria system over the period of incubation) versus time.

It can be seen that there appear to be two distinct types of behaviour. In one case, for the *E. Coli*/MMPP system (Figure 8a), it can be seen that as bacterial kill slowly increases, so too does the intensity of the observed ·OH-adduct signal, reaching a maximum after ca. 700 s. The fact that this maximum broadly coincides

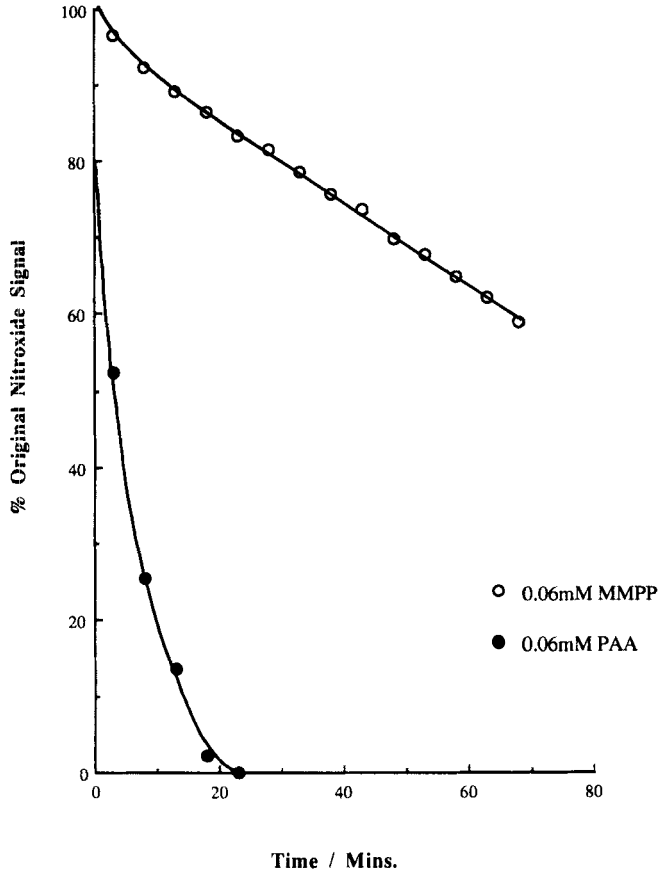


FIGURE 7 Plot of the intensity of the E.P.R. signal of di-t-butyl nitroxide (0.1 mM) versus time in the reaction systems containing *E. Coli* (2×10^9 c.f.u./ml) with either PAA (0.06 mM) or MMPP (0.06 mM).

with the highest % level of bacterial kill offers further evidence that weak bactericides such as MMPP generate hydroxyl radical at a relatively slow rate. In contrast, peracids found to have a strong bactericidal ability, such as PAA and PBTI, appear to produce very rapid initial increases in radical adduct signals – coinciding with rapid bacterial kill – followed by equally marked tail-off, presumably due to radical-radical reactions. For example, for the *Staph. A.*/PAA system (Figure 8b), the % bacterial kill is ca. 90% within 200 s, at which point the observed ·OH-adduct signals are already approaching a maximum value. Similar observations are made for the *Staph. A.*/PBTI spin-trapping system (Figure 8c), where maximum hydroxyl radical signals and ca. 100% bacterial kill coincide after around 250 s incubation.

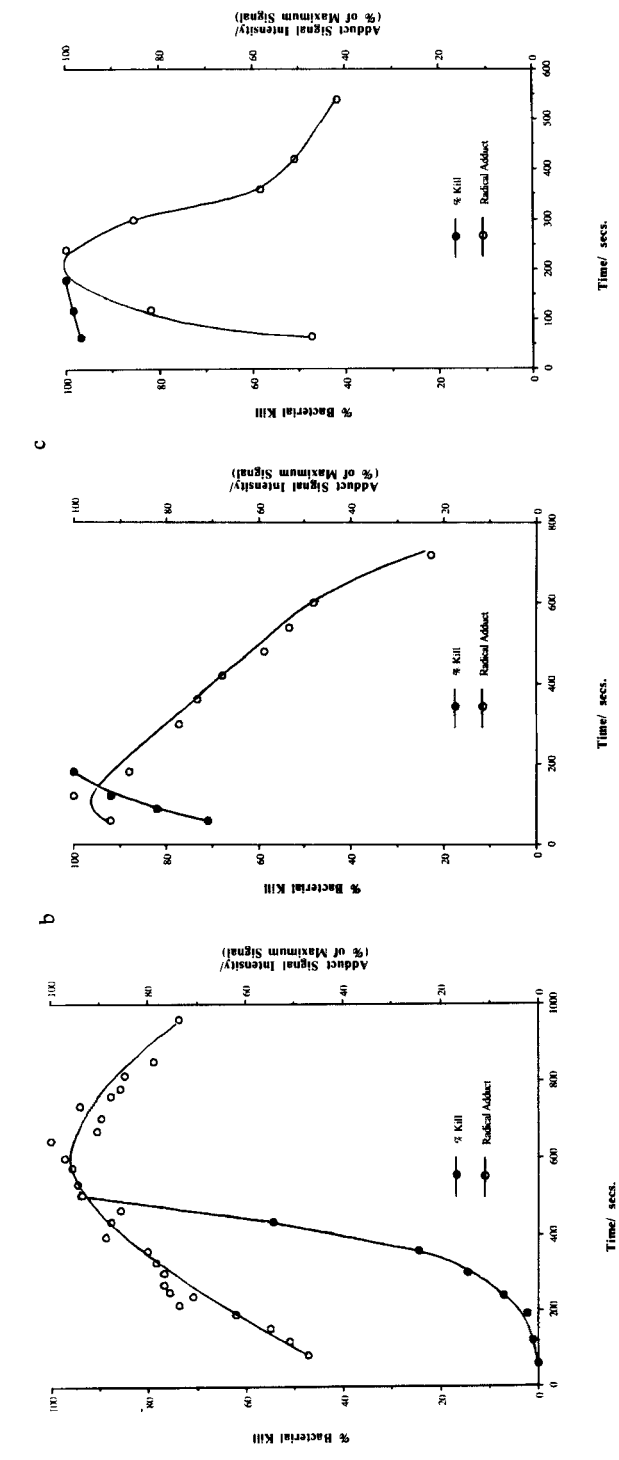


FIGURE 8a,b,c Plot of the intensity of the hydroxyl-radical adduct signal (expressed as a % of the maximum radical adduct observed during the experiment) in a system containing *E. Coli* (2×10^9 c.f.u./ml), DMPO (66 mM) and a) MMPP (0.2 mM); b) PAA (0.2 mM); c) PBT1 (0.4 mM), and the corresponding % bacterial kill in each case for the identical reaction mixture in the absence of the spin trap.

CONCLUSIONS

The EPR spin-trapping experiments reported here demonstrate that both hydroxyl and carbon-centred radicals are produced during the bactericidal action of three peroxide compounds, peracetic acid, magnesium monoperoxyphthalate and 4-percarboxy-N-isobutyltrimellitimide upon both Gram negative (*Escherichia Coli*) and Gram positive bacteria (*Staphylococcus Aureus*). The inhibition of bactericidal action, by DMPO and two antioxidants, Vitamin C and Trolox C, indicates that radicals, which are produced at varying rates for the different bacteria/peracid systems studied, are the major lethal species. Studies of the effect of potassium ferricyanide, a membrane-impermeable paramagnetic broadening agent, upon the observed ·OH-adduct signals suggests that radical production is internal to the bacterial cell. The effect of added iron chelators (which have been shown to protect *E. Coli* and *Staph. A.* from the lethal effects of hydrogen peroxide[1]) and haem-protein inhibitors indicates that iron species (and haem proteins in particular) are at least partially involved in the radical production process. A marked variation is found in radical production both with concentration of peracid and between the three peracids studied. A concentration of peracid is found in each case (the "optimum concentration") at which the observed ·OH-adducts are at a maximum for the peracid/bacteria system. We have found strong inverse correlations for both the value of optimum concentration and the concentration of the observed radical adduct signal in relation to the relative strength of the peroxide as a bactericide. Addition of a stable nitroxide as a radical scavenger has been used to determine relative rates of radical production for different peroxide/bacteria systems; this; confirms that strong bactericides produce radicals at a much faster rate than weak bactericides. The observation that there is a correlation between plots of radical generation and bacterial kill offers further evidence that hydroxyl radicals are the lethal species.

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