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Bactericidal Potency of Hydroxyl Radical in Physiological Environments*

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Rates of radiolytic inactivation of bacteria suspended in N_2O-saturated solutions were dramatically increased over normal background levels when the media contained chloride or bicarbonate ions. The bacteria could be protected from this enhanced toxicity by the addition of free radical scavengers (ethanol, ascorbate, hydrogen peroxide, mannitol, glucose, EDTA, picolinic acid), indicating that the lethal reactions were extracellular in origin. Prior irradiation of chloride-containing solutions led to formation of hypochlorous acid, which was identified by detection of ring-chlorinated products when reacted with fluorescein. Prolonged irradiation of other solutions did not lead to accumulation of bactericidal agents; however, irradiation of bicarbonate-containing solutions in the presence of the spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) led to formation of the EPR-detectable DMPO·CO_3^- adduct. The results are interpreted in terms of formation of secondary radicals, among which the carbonate and chlorine radicals are uniquely toxic to bacteria. From rate comparisons of the solution components, it was concluded that the reactions involving chloride ion are unlikely to be expressed in biological environments, but that the CO_3^- radical could be an important intermediary oxidant in peroxide-inflicted cellular damage, particularly in spatially confined environments such as the leukocyte phagosome.

Cellular damage by hydrogen peroxide is generally regarded as being mediated by transition metal ions (1, 2). Accordingly, the metal ions in their lower valent oxidation states undergo cyclic one or two electron oxidation by H_2O_2, followed by reduction to the original valence state by endogenous reductants or the cellular components themselves. Either the higher valent ions or hydroxyl radical might be the causative agents of cellular damage, although the actual oxidant has not yet been identified conclusively in any physiological system (1–3). A conceptual problem (4) arising in considering these reactions is that, given the extremely reactive character of the hydroxyl radical and higher valent metal ions and the plethora of potential biological reactants, how might selectivity for vulnerable targets within the organism be attained? This problem appears particularly acute for phagocytic cells involved with host resistance, where the reagents for H_2O_2 formation, *i.e.* O_2 and respiratory reductants, could be in limited supply (5, 6). One popular hypothesis is that the metal ions themselves, as catalytic agents for H_2O_2 reduction, confer selectivity on the systems *de facto* by the cellular location of their binding sites (7). In this site-specific mechanism, the powerful oxidants generated are thought to react with biological components in the immediate vicinity of the metal ion, leading to loss of associated cellular function. This mechanism has received widespread acceptance and is supported indirectly by considerable data from various model studies (1, 2, 7).

Consistent with this viewpoint, the deleterious effects of ionizing radiation on cells under most experimental conditions appears to originate from intracellularly generated reactants. For example, survival levels of *Escherichia coli* B following exposure to γ-irradiation were unaltered when high concentrations of polyethylene glycol were included in the reaction medium (8). This polymeric alcohol efficiently scavenges hydroxyl radical formed in the suspending medium but is not taken up by the bacteria. The results imply that hydroxyl radical generated in the external medium by whatever mechanism is an inefficient bactericidal agent. However, when halide ions were present in the medium, radiolytic damage was potentiated by secondary reactions that lead to formation of long lived toxic species, presumably the corresponding halogens or related compounds (9, 10). Biological fluids, of course, contain high concentration levels of chloride and other ions, *e.g.* bicarbonate, which might form secondary radicals from OH which are sufficiently long lived to oxidize vulnerable cellular targets selectively. In this context, bicarbonate has been reported to potentiate protein (11) and amino acid (12) oxidations and enzyme inactivation (13) markedly by OH radicals and to promote photohemolysis of erythrocytes by mechanisms involving undefined activated oxygen intermediates (14).

In this paper, we examine the toxicity of free hydroxyl radicals generated in the extracellular medium toward several bacteria with widely varying envelope composition. Particular attention is given to modeling the reaction environment of the phagocytic cells of the reticuloendothelial system. Here, phagocytosis is accompanied by stimulated respiration involving O_2 reduction to H_2O_2 which is driven by intracellular oxidation of glucose to CO_2 (5, 6). The intraphagosomal chloride concentration in these cells should be very nearly the same as in serum, *i.e.* 0.15 M (15), and the bicarbonate concentration is probably severalfold higher than the usual 25 mM serum levels (15). This environment may therefore represent optimal biological conditions for which cellular damage by externally generated hydroxyl radical could occur.

MATERIALS AND METHODS

E. coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were grown from slant cultures in 0.8% nutrient broth; *Streptococcus lactis* ATCC 7962 was grown from slant culture in 3% trypticase soy broth

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(16). Bacteria were harvested in mid log phase and then washed and suspended to 10^5 cells/ml in the reaction medium. For radiolysis experiments, the suspensions were saturated with N_2O at 4 °C, 3-ml portions transferred to plastic cuvettes, additionally bubbled briefly with N_2O, and capped. The cuvettes were exposed to γ-irradiation from a ^{137}Cs source (20 krad/h) for varying periods of time and the surviving cells determined by their ability to undergo colonial growth using either pour-plate (*E. coli, S. lactis*) or spread-plate (*P. aeruginosa*) methods. In all cases, viabilities were referenced to controls obtained from unexposed cells that were otherwise treated identically to the γ-irradiated samples. Hydroxyl radical formation was estimated by Fe^{2+} dosimetry (17) under standard conditions in cuvettes identical to those containing the bacterial suspensions; quantities of OH radical produced were calculated from the amount of Fe^{3+} formed, determined as its SCN^- complex, and literature G-values (18) for Fe^{2+} oxidation (15.5) and OH formation in anaerobic N_2O-saturated solutions (5.6). All buffers were made from the highest purity commercial salts using reverse osmosis prepurified water that was subsequently glass-distilled or treated in a Barnstead Nanopure II system. Passing reagent solutions through Chelex 100 columns (Bio-Rad) to scavenge adventitious transition metal ion contaminants had no effect upon the experimental results; however, culture-to-culture reproducibilities were high only when glassware and plasticware were acid washed (HNO_3) prior to use. Several trapping experiments were made using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO)[1]; this spin trap (Sigma) was purified by filtering a concentrated aqueous solution through activated charcoal on a Celite mat (19). Room temperature EPR spectra were taken on samples contained in a quartz flat cell using a Varian E-109 X-band instrument; for these experiments, 8–100 mM DMPO was added to the suspension buffers, and spectra were obtained within a few min following γ-irradiation.

To probe the identity of oxidative toxins (9, 10) formed by radiolysis of bactericidal media, parallel studies were made using fluorescein-conjugated polyacrylamide particles. Polyacrylamide spheres having 0.4–1.8-μm diameters were prepared by Dr. Eberhardt Kuhn at Bio-Rad. Fluorescein was covalently attached following procedures developed by Jian-Shen Qi. Briefly, carboxyl end groups were first formed by deamidation in alkaline carbonate buffers (pH 10.5), and then 5-aminofluorescein was linked to the free carboxyl groups using EDC as a coupling agent. As prepared, the particles contained an approximately 30-fold excess of free carboxyl groups over fluorescein; consequently, they were negatively charged under physiological conditions. Detailed preparative procedures and physical characterization of the particles will be reported separately. The fluorescein reporter group could be recovered for chemical analysis as the 5-aminofluorescein by hydrolysis in strongly alkaline solution, followed by neutralization and rotoevaporation of the mixture, then extraction of the dye from the salt residue with methanol. The overall yield following hydrolysis in 1 N NaOH at 95 °C for 3 h was typically 70%.

The compound, *N*-hydroxy-2-thiopyridone (HTP) has been reported to release hydroxyl radical during photodegradation (20, 21). In studies reported herein, we explore its potential use as an alternative source of OH for bactericidal reactions. Bactericidal suspensions containing HTP were photolyzed in 1-cm path length optical cuvettes using a 2,000-watt mercury-xenon lamp. The collimated beam emanating from the lamp housing was passed through Corning O-53 and O-54 cutoff filters to remove wavelengths shorter than 270 nm and through a 10-cm water filter to remove near infrared radiation; relative intensities were varied by placing neutral density filters in the optical path, and sample exposure times were varied with a manually operated shutter. Degradation of HTP was monitored by the loss of its characteristic ultraviolet absorption bands at 330, 280, and 240 nm; optical spectra were recorded on a Hewlett-Packard 8452A diode array spectrometer. Bacterial colony-forming units (cfu) were determined following illumination as described above. The HTP used in these studies was kindly donated by Professor D. H. R. Barton (Texas A & M University).

RESULTS

Cellular Inactivation by γ-Irradiation—Exposure of bacterial suspensions to γ-irradiation in N_2O-saturated buffers led to progressive loss of cfu. The sensitivity to inactivation varied widely with medium conditions and bacterial strain. Representative samples of *E. coli* and *S. lactis* based upon a 7-min bactericidal assay are given in Fig. 1. This exposure time was chosen because it provided optimal discrimination between cidal and non-cidal environments (Figs. 2 and 3). For these organisms, irradiation in phosphate- or sulfate-containing media was ineffective, but irradiation in solutions containing 0.15 M NaCl or 0.1 M bicarbonate buffers caused immediate loss of viability. The effects of 0.1 M HCO_3^- and 0.15 M NaCl were not additive; within experimental uncertainty, no difference was observed in the rate profiles for radiation-induced killing when Cl^- was included in the bicarbonate buffer (Figs. 1 and 3). However, at 25 mM, bicarbonate alone was ineffective, but adding 0.15 M NaCl restored the bactericidal characteristics of the medium (data not shown). Similarly, adding 0.15 M NaCl to phosphate-buffered solutions potentiated their cidal properties under weakly acidic conditions but not under alkaline conditions (Figs. 1 and 3). Adding NaCl did not potentiate killing in 0.1 M Na_2SO_4 (Figs. 1 and 3), but the full bactericidal effects of the Cl^- ion were expressed when the concentration of sulfate was reduced to 5 mM (data not shown).

E. coli and *S. lactis* viabilities in cidal environments are displayed in Figs. 2 and 3, respectively, as a function of dose level. These sigmoidal curves are typical of the cfu *versus* irradiation plots that formed the basis for the data summarized in Fig. 1; the dose response under a given set of reaction condi-

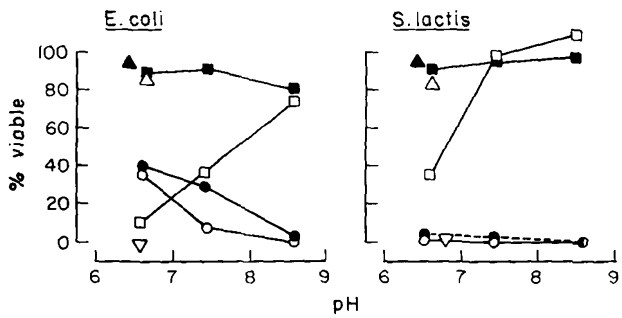


FIG. 1. **Surviving cells following 7-min exposure to γ-irradiation.** Results are for 6 × 10^5 cfu in 3 ml of various media. For this and Figs. 2–4, all *open symbols* refer to media to which 0.15 M NaCl has been added, and all *solid symbols* refer to media containing no added chloride ion. ▲, 0.1 M Na_2SO_4; ■, 0.1 M NaH_2PO_4/Na_2HPO_4; ●, 0.1 M $NaHCO_3/CO_2$; ▽, 0.15 M NaCl alone.

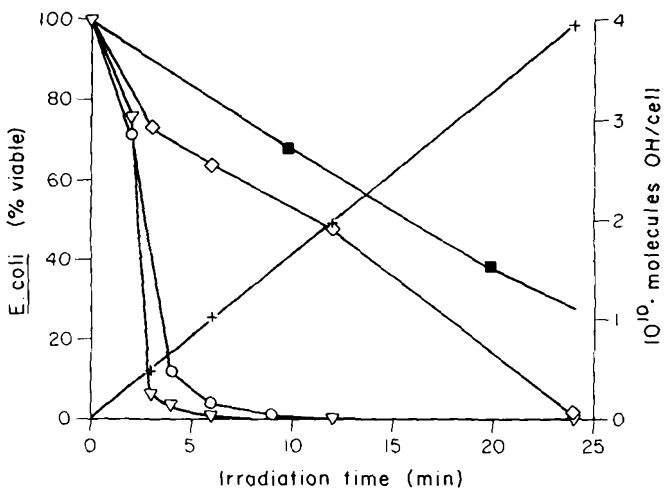


FIG. 2. **Radiolytic inactivation of *E. coli*.** Results are for 10^6 cfu in 3 ml of N_2O-saturated 0.15 M NaCl, pH 6, without additions (▽) or containing 70 mM ethanol (◇), 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.5 (■), or 0.15 M NaCl + 25 mM $NaHCO_3$, pH 8.7 (○). Hydroxyl radical yields (+) determined by Fe^{2+} dosimetry as described under "Materials and Methods."

[1] The abbreviations used are: DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HTP, *N*-hydroxy-2-thiopyridone; cfu, colony-forming unit(s); HPLC, high performance liquid chromatography; T, tesla.

tions was highly reproducible. Also shown in Fig. 2 is the protective effect of ethanol, an OH radical scavenger (18). At 70 mM, ethanol gave equivalent protection (70–100% at 7-min exposure) in all of the bactericidal media given in Fig. 1; the extent of protection was independent of the ethanol concentration over the experimental range examined (for *E. coli*, 70–280 mM; for *S. lactis*, 1–50 mM). Other OH-reactive compounds (18) were also protective. Specifically, the following compounds (which were tested at the indicated concentration levels) gave the same protection as ethanol: for *E. coli*, 0.5 M mannitol, 1.0 mM EDTA, 0.5 mM picolinate ion, 4 mM glucose (in 5 mM phosphate, 0.15 M NaCl, pH 7.4), 10–50 μM ascorbate, and 11–1070 μM H_2O_2; for *S. lactis*, 0.5 mM mannitol, 1.0 mM EDTA, 10–50 μM ascorbate, and 10–100 μM H_2O_2. Nonetheless, slow cellular inactivation occurred even in the presence of these agents, as was apparent from exposure of the suspensions to longer periods of irradiation. This effect was more pronounced for *E. coli*, where radiolysis in 0.1 M phosphate buffer, pH 7.4, or in the presence of 70 mM ethanol (Fig. 2) for 30 min gave essentially complete loss of cfu. For *S. lactis*, 75% of the cells retained viability under comparable conditions (*cf.* Figs. 2 and 3).

P. aeruginosa exhibited a different response to irradiation. As with the other organisms, no loss of cfu was observed in phosphate-buffered solutions upon 6-min irradiation; however, losses in 0.15 M bicarbonate and/or 0.15 M NaCl were approximately 50% over that period, and the cells could not be protected from inactivation by addition of ethanol.

Detection of Toxic Intermediates—To examine whether or not the bactericidal properties of γ-irradiated solutions could be attributed to formation of a persistent toxin, experiments were conducted in which 2×10^5 cfu/ml *E. coli* or *S. lactis* suspensions were added to previously irradiated solutions. Killing was complete when 0.15 M NaCl irradiated for 60 min was mixed with the bacteria, providing that the time interval between irradiation and mixing was short. When the time interval exceeded 25 min, the toxicity of the irradiated solutions was significantly diminished. Also, killing was less effective in more acidic saline solutions, *i.e.* pH 5.0 *versus* 6.6. In other media, including 0.1 M bicarbonate or 0.15 M NaCl in 0.1 M bicarbonate and phosphate buffers, pH 7.4, no killing was observed after prior irradiation of the solutions for 60 min.

γ-Irradiated unbuffered 0.15 M NaCl solutions oxidized *N,N*-diethyl-*p*-phenylenediamine to a red product. Although this dye is used in standard assays for chlorine, it is reactive toward other strong oxidants, including hydrogen peroxide (22), which can be formed by combining two OH radicals (18). A more diagnostic dye is fluorescein, which undergoes ring chlorination primarily at the 4′- and 5′-positions upon reaction with HOCl (23) as shown in Scheme 1. The chlorinated fluoresceins are distinguishable by their characteristically red shifted optical absorption and fluorescence emission bands; fluorescence quantum yields are also lower in the chlorinated dyes (23). The addition of fluorescein-conjugated polyacrylamide beads to saline solutions that had been irradiated for 70–90 min gave immediate formation of compounds with red shifted bands and 30–40% overall loss in fluorescence intensity. When measured in 0.1 M phosphate buffer, pH 7.4, the fluorescence excitation and emission band maxima were found at 494 and 519–21 nm, respectively, in the fluoresceinated particles exposed to irradiated solutions, compared with 491 and 517 nm for the original samples. These properties are consistent with extensive monochlorination of the fluorescein ring (23). Structural confirmation was obtained by hydrolytic solubilization and HPLC analysis of the dye. Approximately 10-fold greater number densities of the particles were used in these experiments. Chromatograms of 5-aminofluorescein isolated from particles exposed to irradiated solutions exhibited two new bands, each with about 10% of the area of the recovered unmodified dye. One of these species coeluted with authentic samples of 4′-chloro-5-amino-

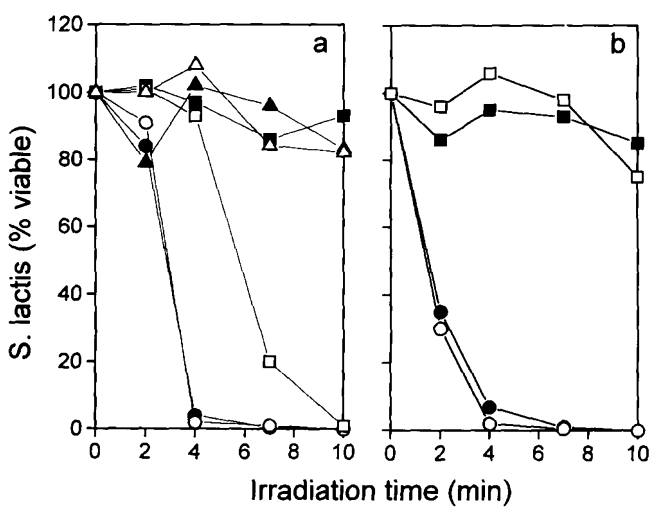


FIG. 3. **γ-Irradiation of 2×10^5 cfu/ml *S. lactis* in various buffers at pH 6.6 (*panel a*) and pH 7.4 (*panel b*).** *Symbols* are as defined in Fig. 1. The *ordinate* is the percent survival based upon unirradiated controls.

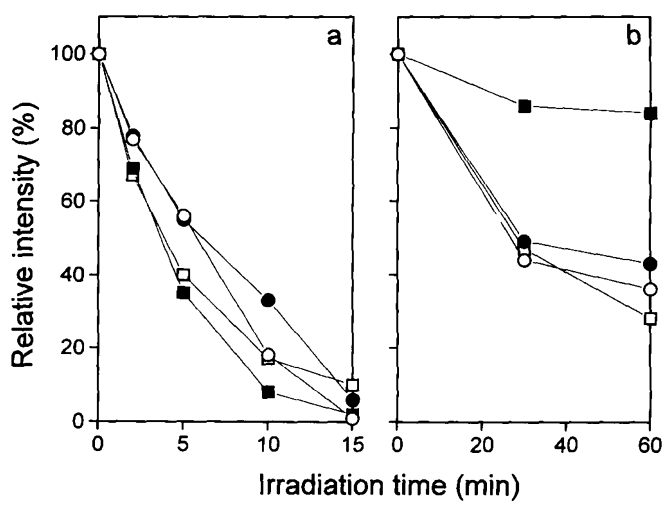
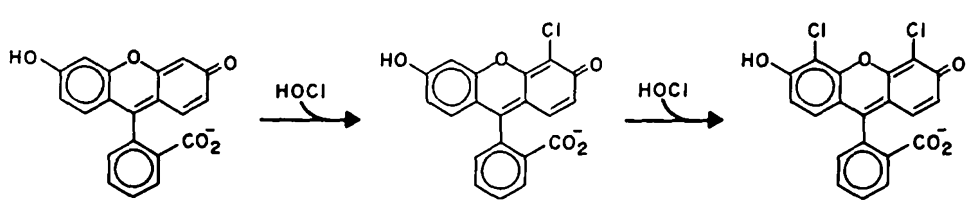


FIG. 4. **Loss of fluorescence intensity upon γ-irradiation of 2 μM fluorescein (*panel a*) or particle-bound fluorescein (*panel b*) in various media.** Symbols are as defined in Fig. 1. For fluorescein, excitation was at 490 nm, and emission intensities were measured at 515 nm; slit widths were 2.0 and 1.6 nm, respectively. For the particles, excitation and emission wavelengths were 492 and 516 nm, with both slits set at 6.0 nm.

SCHEME 1.



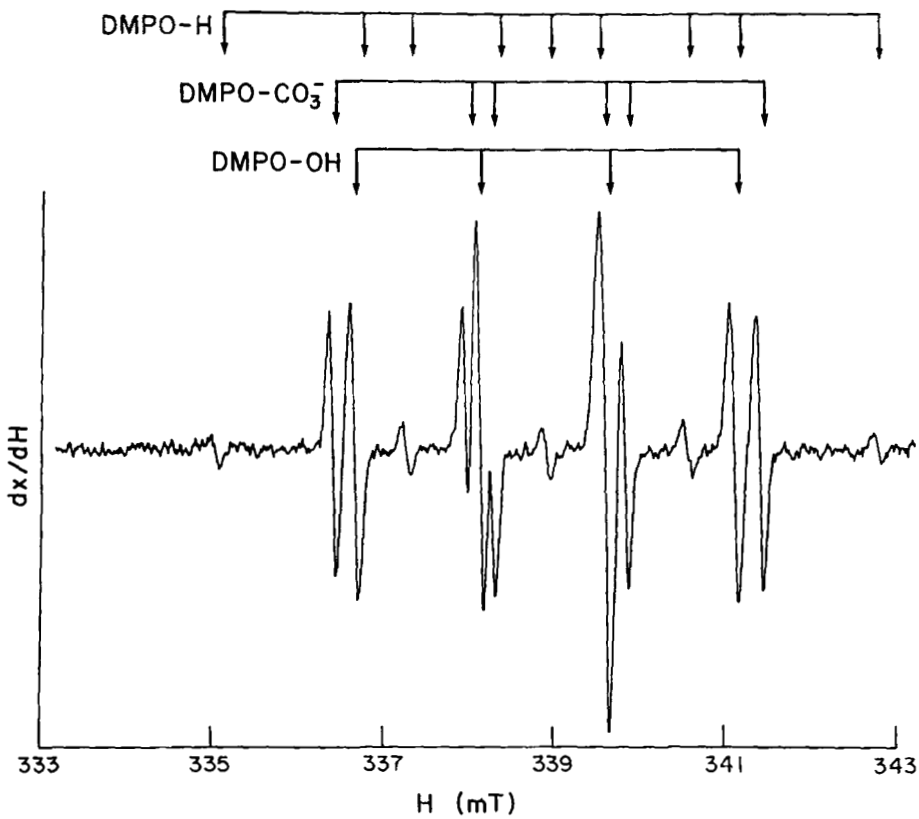


FIG. 5. **DMPO EPR spectrum after γ-irradiation in HCO_3^-/CO_2 buffer.** Conditions: 10 mM DMPO, 200 mM $NaHCO_3$, pH 6.6, irradiated 10 min. Spectral parameters: 9.530 GHz microwave frequency; 20 milliwatts microwave power; 296 K; 0.1 mT modulation amplitude; 5 mT/min scan rate; 0.128 s time constant. *Arrows* indicate band assignments for the various DMPO spin adducts.

fluorescein; the second band could not be identified by comparison with available isomeric mono- and dichlorofluoresceins. However, both 4′-chloro-5-aminofluorescein and the unidentified species were formed when 5-aminofluorescein was reacted with a stoichiometric amount of HOCl in 0.1 M phosphate, pH 4–7, containing 0.15 M NaCl. 4′-Chloro-5-aminofluorescein and 4′,5′-dichloro-5-aminofluorescein were the only detectable products when 5-aminofluorescein or the fluoresceinated particles were reacted with HOCl in the absence of added NaCl. Analogous behavior was observed when fluorescein was used in place of 5-aminofluorescein. Thus, particle-bound fluorescein reacted with irradiated phosphate-buffered saline solutions to give chlorinated products that were equivalent to those formed when fluorescein or 5-aminofluorescein reacted with HOCl in phosphate-buffered saline.

Radiolysis of solutions containing 2 μM fluorescein caused extensive bleaching of its optical bands *without* inducing any perceptible wavelength shifts in the remaining chromophore. This reaction was insensitive to the solution identity; particularly noteworthy was the absence of rate-promoting effects when chloride ion was present in the medium (Fig. 4*a*). In contrast, the fluorescein-conjugated particles were much less reactive under most conditions, but rates of oxidative degradation increased when 0.15 M NaCl or 0.10 M $NaHCO_3$ was added (Fig. 4*b*). Fluorescence excitation and emission spectra underwent progressive red shifting from 492 to 502 nm and from 516 to 528 nm, respectively, over the course of the reaction when the medium contained only chloride ion; when either HCO_3^- alone or both HCO_3^- and Cl^- were present, chromophoric bleaching of the fluoresceinated beads was not accompanied by shifts in the positions of the spectral band maxima.

Under most conditions, γ-irradiation of DMPO-containing suspension media gave EPR spectra containing a strong DMPO·OH adduct signal (24, 25) ($A_H = A_N = 1.48$ mT) superimposed upon a very weak signal with $A_N = 1.62$ and $A_H = 2.2$ mT, the latter exhibiting a 1:2:1 splitting pattern attributable to two equivalent hydrogen atoms which identifies the compound as the DMPO·H adduct (25). However, in HCO_3^- buffer below pH 7.2, an additional signal with $A_N = 1.57$ mT, $A_H = 1.85$ mT was detected, which became relatively more intense upon acidification to pH 6.2 (Fig. 5). These hyperfine splitting constants are nearly identical to reported values for the formate radical adduct ($A_N = 1.56$ mT, $A_H = 1.87$ mT) (26); therefore, this compound is most likely the carbonate radical adduct.

Photolytic Generation of Hydroxyl Radical—For the photolysis experiments, source intensities were usually adjusted to give an initial HTP decomposition rate of about 0.3 mM s^{-1}. These experiments were usually carried out in 0.1 M phosphate buffer, pH 6.5, containing 0.15 M NaCl, which is bactericidal in the presence of ionizing radiation (*cf.* Fig. 1). To determine whether hydroxyl radical was produced during photolysis, spin trapping experiments were undertaken. The DMPO·OH adduct was observed when solutions containing 1 mM HTP and 100 mM DMPO were photolyzed, but no radicals could be detected at higher HTP concentrations (10–100 mM). This result suggests that HTP and/or its photolysis products can also scavenge OH radical in competition with DMPO. HTP was also found to protect *E. coli* from γ-irradiation in a manner analogous to that displayed in Fig. 2. Specifically, when 10 mM HTP was added to N_2O-saturated buffer suspensions containing 2×10^5 cfu/ml *E. coli*, the rate of killing fell to levels that were identical to the background rate observed in the presence of OH scavengers; 4 mM DHP afforded about 50% protection of the cells under these conditions. Given this protective effect, the photolysis experiments were conducted by adding HTP repetitively in small aliquots over the reaction course with intermittent exposure to the light beam between additions. Each increment constituted one-tenth of the total added HTP, and exposure time intervals were 5 s. The total added HTP ranged from 0.15 to 10 mM; its effects upon both *E. coli* and *S. lactis* were examined. Typical results are tabulated in Fig. 6. The data were complicated by significant background killing rates arising from illumination

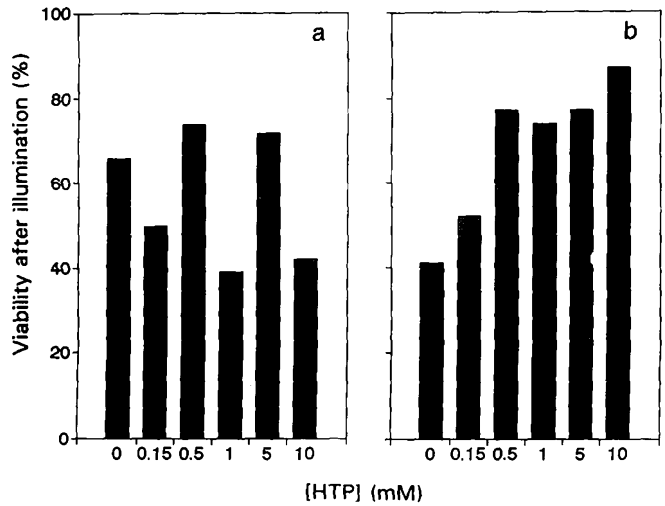


FIG. 6. **Illumination of bacterial suspensions.** *Panel a, E. coli; panel b, S. lactis.* 2×10^5 cfu/ml in 0.1 M phosphate, pH 6.5, containing 0.15 M NaCl was illuminated in 10 5-s intervals adding one-tenth of the total indicated HTP between illumination periods. Experimental details are given under "Materials and Methods."

with the filtered light alone, which was approximately 50% for both organisms under these conditions. Nonetheless, no additional killing was evident for *E. coli*, and higher concentrations of HTP appeared to protect *S. lactis* from light-induced damage, most likely through an inner filter effect. Several experiments were also made with *S. lactis* at reduced light levels where HTP photodegradation was still substantial, but background inactivation of the cells by ultraviolet irradiation was negligible. The addition of 0.025–5.0 mM HTP in 10-ml aliquots separated by 10-s exposure times did not elicit any significant cellular inactivation, either in 0.1 M bicarbonate, pH 7.5, or in 0.1 M phosphate, pH 6.5, plus 0.15 M NaCl.

DISCUSSION

Reaction Dynamics—The effects of hydroxyl radical scavengers on cellular viabilities in the presence of ionizing radiation can be used as a criterion for defining two distinct bactericidal mechanisms (8, 27). Specifically, the scavengers were unable to protect the bacteria from lethal doses of γ-irradiation in media that did not contain Cl^- or HCO_3^- ions. In this case, either (i) the toxins were generated at sites that were inaccessible to the scavengers, *i.e.* within the cellular envelope, and reactions initiated by extracellularly formed OH were ineffective; or (ii) the bacteria competed effectively for OH generated in the suspending medium under all conditions. Relative rate arguments (discussed below) indicate that the first explanation must be correct. However, *E. coli* and *S. lactis* could be protected from the enhanced killing observed at high saline or bicarbonate concentrations (Fig. 2). It follows that the enhanced killing must have arisen from reactions initiated in the suspending medium. Therefore, these systems present an opportunity to investigate the potential toxicity of extracellularly generated OH radical under conditions that mimic biological environments.

Radiolysis of N_2O-saturated solutions produces OH radicals and H atoms as the only free radical species; OH is the predominant product, comprising about 90% of the total radical yield (18). Hydroxyl radical can undergo hydrogen atom abstraction or electron transfer reactions with each of the anions used in these studies to form new radical species. These reactions and relevant rate parameters are listed in Table I. Although the reaction with bicarbonate ion is relatively fast, they are all considerably slower than the reactions of OH with most simple organic compounds and ions (18), many of which approach diffusion-controlled limits ($k \simeq 10^{10}$ M^{-1} s^{-1}). One might, therefore, question whether these anions could compete with the bacteria for OH radicals that were radiolytically generated in the suspending medium. Based upon standard treatments of diffusion-controlled processes, the maximal rate constant calculated for reaction of OH with a bacterium is $k_D \simeq 4 \times 10^{14}$ M^{-1} s^{-1}; this unusually large value reflects the bacterial size (1 μm). At the cell densities used in these experiments ($\leq 10^6$ cfu/ml), the total excluded volume occupied by the bacteria is negligible ($<4 \times 10^{-4}$% of the solution volume). Consequently, they can be approximated as molecular entities with respect to OH radicals that are randomly generated in the extracellular medium. The effective concentration of 10^6 bacteria/ml is about 10^{-16} M. The relative extent of reaction by competing (or parallel) pathways is given by the ratio of the relative rates (R), hence, the ratio of products of rate constants and concentrations. Rate ratios calculated for all of the experimental conditions used in this study indicate that direct reaction of extracellularly generated OH radicals with the bacteria should be negligible. For example, in 0.15 M NaCl, the calculated rate ratio is $R_{\text{bacteria}}/R_{Cl^-} \leq k_D[\text{bacteria}]/k_4[Cl^-] \leq 3 \times 10^{-4}$. Similarly, relatively low concentrations of scavenger ions are predicted to be protective; for example, calculated rate ratios in the presence of 10 μM H_2O_2 are $R_{\text{bacteria}}/R_{H_2O_2} \leq k_D[\text{bacteria}]/k_8[H_2O_2] \leq 2 \times 10^{-4}$, where $k_5 = 2.2 \times 10^7$ M^{-1} s^{-1} for the following reaction (28).

$$OH + H_2O_2 \rightarrow H_2O + H^+ + O_2^- \qquad \text{(Reaction 8)}$$

The low rates of reaction of OH radicals with bacteria in dilute suspensions are because of spatial effects. Although the bacteria are made up of OH-reactive biomolecules, these are isolated within very small elements of the total solution volume. Consequently, the mean diffusion distance for OH radicals to reach a target site within the bacterium is much longer than if its component biomolecules were distributed uniformly throughout the solution, and the reaction rate is correspondingly slower. This effect is illustrated experimentally by the radiolytic reactions of fluorescein. In homogeneous solution, fluorescein was oxidized rapidly in reactions that were independent of

TABLE I
Secondary reactions of OH radical with medium components at pH 7

Reaction	Rate constant	Ref.
	$M^{-1} s^{-1}$	
(1) $OH + HCO_3^- \rightarrow H_2O + HCO_3$	8×10^6	33
(2) $OH + SO_4^{2-} \rightarrow OH^- + SO_4^-$	$<10^4$	45[a]
(3) $OH + H_2PO_4^- \rightarrow H_2O + PO_4^-$	2×10^6	43
(3′) $OH + HPO_4^{2-} \rightarrow H_2O + PO_4^{2-}$	8×10^5	43
(4) $OH + 2Cl^- + H^+ \rightarrow H_2O + Cl_2^-$	1×10^3	44[b]

[a] The SO_4^- radical was detected as a transient species following pulse radiolysis of acidic solutions but could not be detected in neutral solutions containing the SO_4^{2-} ion; the reaction half-time ($t_{1/2}$) for radical decay was about 20 μs (45). The decay rate can be used to estimate an upper limit for the rate constant for reaction between the SO_4^{2-} ion and OH radical since the condition that precludes SO_4^- radical accumulation is that its rate of formation is less than its decay rate. Assuming $t_{1/2} \geq 60$ μs for SO_4^- formation in 1 M Na_2SO_4 (45), the corresponding rate constant is $k \leq 1.2 \times 10^4$ M^{-1} s^{-1}.

[b] This reaction is complex and has been proposed to involve the intermediacy of $HOCl^-$, which decomposes to Cl and H_2O upon protonation, followed by association with Cl^- to give Cl_2^- (30) *i.e.*

$$OH + Cl^- \rightleftharpoons HOCl^- \qquad \text{(Reaction 5)}$$
$$H^+ + HOCl^- \rightleftharpoons H_2O + Cl \qquad \text{(Reaction 6)}$$
$$Cl + Cl^- \rightleftharpoons Cl_2^- \qquad \text{(Reaction 7)}$$

Although each of the steps is nearly diffusion-controlled, conversion of $HOCl^-$ to Cl requires a proton; under physiological conditions, this step will be slow and yields an apparent rate constant for reaction 4 at pH 7 of $k_4' \simeq 1.3 \times 10^3$ M^{-1} s^{-1}, based upon reported rate constants. The radical intermediates $HOCl^-$ and Cl do not accumulate under these conditions (30).

medium composition (Fig. 4*a*); bicarbonate, if anything, was protective, and no chlorinated products were formed, suggesting that fluorescein had reacted directly with OH radical. However, when the fluorescein was confined to particles whose size was the same as bacteria, basal reaction rates decreased dramatically, and reactivity was enhanced by adding HCO_3^- or Cl^- salts to the medium (Fig. 4*b*), analogous to the bactericidal reactions. Spectroscopic shifts indicating that ring chlorination had occurred in bicarbonate-free saline solutions established that the reactions of the particle-bound fluorescein were indeed mediated by secondary radicals.

One concludes that almost all (*i.e.* >99.99%) of the extracellularly generated OH radicals reacted with medium components, rather than directly with the bacteria under these conditions. Furthermore, most secondary radicals formed by one-electron reduction of OH, including reactions with phosphate and sulfate anions and the various added hydroxyl radical scavengers and electron donors, were not toxic to the bacteria. Exceptions were the radicals formed in aqueous saline solutions and bicarbonate buffers. The reactivity patterns observed for more complex solutions containing several components can also be rationalized with the conceptual model given above. Specifically (i) bacteria could be protected from the enhanced toxicity by low concentrations of radical scavengers, *e.g.* 10 μM H_2O_2 or ascorbate, confirming that the bactericidal reactions were relatively slow. (ii) Phosphate anions at alkaline pH and SO_4^{2-} reversed the effects of added Cl^- ion (Figs. 1–3), consistent with relative magnitudes of their rate constants with OH radical (Table I). (iii) The spectral changes observed in fluoresceinated particles suspended in media containing nearly equimolar HCO_3^- and Cl^- ions were consistent with reaction with bicarbonate-derived, but not chloride-derived, intermediates, consistent with the much greater rate of HCO_3^- ion with the OH radical (Table I). (iv) Spin trapping with DMPO gave evidence for formation of additional medium-derived radicals only with HCO_3^- ion, which is the only electrolyte anion that reacts rapidly enough to compete with DMPO for OH radical.

Identities of the Bactericidal Species—Enhanced toxicity in N_2O-saturated phosphate-buffered saline has been reported previously (9, 10) and was suggested by Brustad and Wold (9) to arise from accumulation of Cl_2 and HOCl during radiolysis. Our results using fluorescein as a probe provide direct evidence for formation of chlorine compounds under these conditions; specifically, in addition to the diagnostic chloride-specific spectral shifts noted above, chlorinated products were identified by HPLC analysis of the dye recovered from particles that were exposed to γ-irradiated 0.15 M NaCl solutions. Hypochlorous acid could be formed by radical recombination of Cl_2^-, followed by hydrolysis of intermediary Cl_2, *i.e.*

$$2Cl_2^- \rightarrow Cl_2 + 2Cl^- \qquad \text{(Reaction 9)}$$

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \qquad \text{(Reaction 10)}$$

or directly by radical combination reactions, *e.g.* $OH + Cl_2^- \rightarrow HOCl + Cl^-$. The apparent toxicity of OH in Cl^--containing media towards *S. lactis* and *E. coli* is remarkably high, requiring less than 10^{10} OH radicals/bacterium to achieve 99% reduction in cfu (Fig. 2). This number can be compared to about 10^9 HOCl/*E. coli* to reach equivalent inactivation under similar conditions (29). Thus, assuming HOCl is the toxin, about 10% of the initially formed OH is converted to hypochlorous acid. However, the G-value for Cl_2^- formation in 0.1 M neutral NaCl solution is reported (30) to be 0.17, which is only 3% of the G-value for OH formation. Thus, to account for the high toxicity of γ-irradiated Cl^--containing solutions, some additional mechanism must be contributing to HOCl formation. Brustad and Wold (9) have suggested that N_2O can participate by reacting with the transient species (see Footnote *b* in Table I), $HOCl^-$, to give HOCl and regenerate OH.

$$H^+ + HOCl^- + N_2O) \rightarrow HOCl + N_2 + OH \qquad \text{(Reaction 11)}$$

Reaction 11 is appealing because in combination with Reaction 5 (see Footnote *b* in Table I) it establishes a chain reaction whose overall stoichiometry is $Cl^- + H^+ + N_2O \rightarrow HOCl + N_2$; this reaction is exothermic by 0.29 V at pH 7 (31, 32).

Unlike chlorine radicals, complications arising from reaction of HCO_3 with N_2O must be insignificant because further oxidation of the carbonate radical is energetically prohibitive. Consequently, the enhanced toxicity of bicarbonate-containing solutions is probably attributable to direct reaction of the bacteria with carbonate radicals formed from OH (Reaction 1). This suggestion is supported by the following arguments. (i) Reaction 1 is well documented (33–35), and HCO_3 formation was demonstrated under our conditions using DMPO spin trapping methods. (ii) Prolonged irradiation of 0.1 M bicarbonate buffers did not cause formation of persistent toxins, consistent with radical species being the bactericidal agent(s). (iii) The apparent increased reactivity reported for OH toward proteins in bicarbonate buffers (11–13) indicates that these radicals are potentially lethal. (iv) HCO_3, by virtue of its relatively long lifetime and low reactivity (36), possesses the essential requirements of an effective bactericidal agent (5). Decay of HCO_3 is bimolecular, with a rate constant $k = 1.5 \times 10^7$ M^{-1} s^{-1} (34, 35). Based upon Fe^{2+} dosimetry, the rate of OH production by radiolysis was 1.6×10^{-8} Ms^{-1} (Fig. 2); equating this with the rate of HCO_3 formation in bicarbonate buffers, the calculated steady-state concentration level of HCO_3 is 32 nM, giving an intrinsic lifetime (τ) of 2.1 s, for which the average distance of diffusion, calculated from $(\overline{\Delta X^2})^{1/2} = (2D\tau)^{1/2}$ with $D = 10^{-5}$ cm^2 s^{-1}, is estimated to be ~65 μm. The average separation distance of 3×10^5 bacteria/ml is ~150 μm, so the maximum diffusion distance for a HCO_3 radical to a bacterial surface is ~75 μm. Therefore, under these conditions, most of the HCO_3 radicals generated in the suspending medium will be able to reach bacterial target sites. In contrast, the average distance of diffusion of the OH radical in the various ionic media, calculated using the rate constants listed in Table I, is $(\overline{\Delta X^2})^{1/2} = 0.05$–3.7 μm. Thus, only the OH radicals generated very near bacterial surfaces will survive to react with the cells, a conclusion that is consistent with the previously discussed relative rate analyses. Several studies have shown that rate constants for reaction of HCO_3 radical with amino acids, dipeptides, and related compounds vary widely; among the amino acids, appreciable reactivity was observed only for tryptophan, tyrosine, histidine, and the sulfur-containing compounds (37, 38). Based upon measured rates of enzyme inactivation and spectral detection of radical intermediates, these amino acids are apparently also selectively oxidized in proteins (37, 39). Furthermore, reactivities of OH radical scavengers such as alcohols, glucose, and urea are 10^5–10^7-fold lower toward HCO_3 (37, 40). Thus, carbonate radical has reactivity characteristics appropriate for selective oxidation of a limited number of cellular sites, which may optimize the probability of generating lethal lesions, as required for an effective toxin (5).

Physiological Significance—Enhancement of OH toxicity by bicarbonate and chloride ions might have biological purpose in host resistance to disease, specifically involving phagocytic cells of the reticuloendothelial system. Plasma concentration levels of chloride and bicarbonate ions are 0.15 M and 25 mM, respectively (15). The value of HCO_3 is undoubtedly a lower limit at sites of infection since the respiratory burst accompanying foreign body recognition by phagocytic cells generates CO_2 (5, 6). The other respiratory product is superoxide ion,

which disproportionates in near quantitative yield to form H_2O_2 (41). *If* H_2O_2 underwent subsequent metal-catalyzed one-electron reduction within the phagosomal milieu, conditions would exist for secondary generation of the carbonate or chloride ion-derived bactericidal agents from hydroxyl radical. Alternatively, other oxidizing intermediates formed in metal-catalyzed peroxide reactions, *e.g.* Cu(III) or Fe(IV) species, might have sufficient thermodynamic potential to directly oxidize bicarbonate ion to HCO_3. The phagosomal medium is much more complex than our model environments, however, and contains many additional components capable of reacting with OH-derived radicals. For example, 10 μM H_2O_2, a concentration probably exceeded within phagosomes during stimulated respiration (41, 42), protected bacteria against chloride and bicarbonate-mediated killing in our systems. Numerous other OH-reactive compounds were shown to be equally effective, including both HTP, which actually generated OH photolytically but protected bacteria from its lethal effects, and the organic electron donors EDTA and picolinate, which are often used as ligands in cell-free metal catalyzed oxidation reactions designed to mimic biological H_2O_2-initiated degradation reactions. Previous workers have also reported that the toxicity-enhancing effects of chloride ion toward *E. coli* could be reversed by adding 500 μM valine or cysteine (10).

The biological significance of the enhanced toxicity of radiolyzed saline solutions is difficult to assess because the extent of contributions from nonphysiological reactions of $HOCl^-$, Cl or Cl_2^- with N_2O (*e.g.* Reaction 11) is unknown. However, relative rate comparisons similar to those described above suggest that Cl_2^- formation from OH radical must be minimal in biological environments because competitive reactions would undoubtedly predominate. These kinetic restrictions do not apply to bicarbonate ion, which is about 10^4 times more reactive than Cl^- toward OH radical. When $[HCO_3^-] \simeq 0.1$ M, relative rate calculations predict nearly quantitative conversion to the carbonate radical even in the presence of high concentrations of OH-reactive biomolecules.[2] Thus, the question of whether or not HCO_3^- can mediate OH toxicity through HCO_3 formation depends upon the extent to which competitive reactions with medium components protect the bacteria. Our experiments show that, in dilute suspensions, reactions with radical scavengers predominate, as might be expected from calculations similar to those described above for OH reactivity. However, in the confined environment of the phagosome, where the effective bacterial "concentration" is much larger simply because it occupies most of the intraphagosomal space, the bacterium can be expected to compete effectively with reactants in the phagosomal fluid for the HCO_3 radical. This is a second type of spatial effect in which reaction with large particles is promoted, rather than retarded, relative to molecular species present in the surrounding medium; the effect is readily demonstrated using a quantitative model that considers the phagosomal size, oxidant diffusion rates, and reported rate constants for biological components with OH and HCO_3 radicals.[3] The confinement might be considered tantamount to site directedness. It is distinct from site-specific mechanisms (1, 2, 7), however, in proposing that, irrespective of the topographic origin of hydroxyl radical, its toxicity is enhanced by formation of secondary radicals from medium components, *i.e.* HCO_3^- ions. In any event, the remarkable enhancement of radiolytic damage by Cl^- and HCO_3^- ions in simple solutions suggests that their potential influence should be considered when investigating biological free radical and related oxidative reactions. A hierarchy of the reactions of OH radical based upon these rate comparisons is given schematically in Fig. 7.

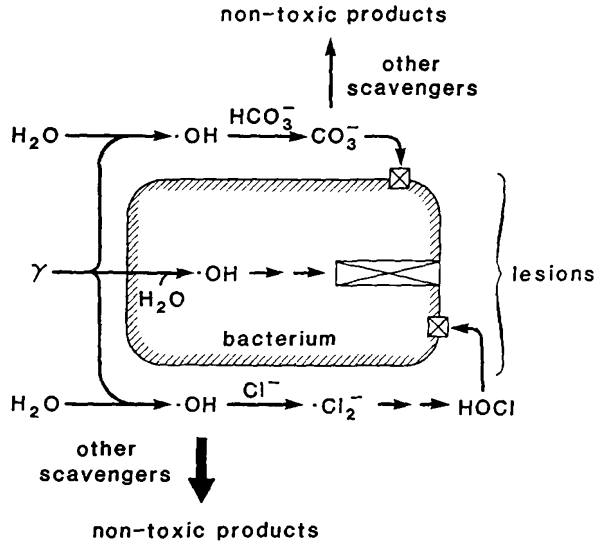


FIG. 7. **Hypothetical mechanisms for Cl^- and HCO_3^--enhanced radiolytic killing of a bacterium.** Extracellular Cl_2^- radical formation is suppressed by OH-reactive compounds in the medium, but extracellular CO_3^- radical formation is not. In this case, protection by free radical scavengers must arise by direct competition for the CO_3^- radical. The reaction scheme includes an intracellular pathway, although cytosolic target sites are not specified.

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[2] For example, assuming that the cumulative concentration of OH-reactive biomolecules whose rate constants are $k = 10^8$ M^{-1} s^{-1} is 1 mM, $R_{HCO_3^-}/R_{other} \simeq k_1[HCO_3^-]/(10^8 \cdot 10^{-3}) \simeq 8$ when $[HCO_3^-] = 0.1$ M, so that ~90% of the OH consumed yields HCO_3 radical.

[3] S. V. Lymar and J. K. Hurst, manuscript in preparation.

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