Kinetics of Paraquat and Copper Reactions with Nitroxides: The Effects of Nitroxides on the Aerobic and Anoxic Toxicity of Paraquat

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The toxicity of paraquat (PQ^{2+}) is attributed to intracellularly formed $PQ^{\bullet+}$, $O_2^{\bullet-}$, H_2O_2, and secondary ·OH radicals generated through Fenton-like reactions. Yet, no antidote for PQ^{2+} toxicity in human has been found also due to poor cell permeability of many common antioxidants that remove toxic species predominantly extracellularly. Cell-permeable nitroxides, which scavenge xenobiotic-derived deleterious radicals and detoxify redox-active metal ions, would be expected to ameliorate PQ^{2+} toxicity. We have studied using pulse radiolysis the kinetics of the reactions of 2,2,6,6-tetramethyl-piperidinoxyl (TPO) and 4-OH-TPO with $PQ^{\bullet+}$ and $Cu^{II}L_2$ (L = 1,10-phenanthroline, 2,2′-bipyridyl) in the absence and presence of DNA. We found that the rate constant for the reaction of $PQ^{\bullet+}$ with the nitroxides is about 4 orders of magnitude lower than that with O_2. In addition, the rate of the reaction of the nitroxides with $Cu^{I}L_2$ decreases as [DNA] increases, which suggests that nitroxides react significantly slower with bound metal ions. These results explain the failure of 4-OH-TPO to protect bacterial and mammalian cells from PQ^{2+} toxicity under air. In contrast, the rate of the reaction of $PQ^{\bullet+}$ with $Cu^{II}L_2$ was unaffected by DNA. Furthermore, copper toxicity has been attributed mainly to Cu^{I} and was observed predominantly for cells subjected to anoxic conditions. It implied that nitroxides would be effective protectants if PQ^{2+} induces toxicity also under anoxia. Surprisingly, we found that PQ^{2+} toxicity under anoxia was even greater than that under air, and under these conditions 4-OH-TPO protected the cells from PQ. These results indicate that the mechanism underlying the anoxic toxicity of PQ^{2+} differs from that operating in the presence of oxygen, and that reduced transition metal ions are most probably the species responsible for PQ^{2+} anoxic toxicity.

Introduction

The chemistry and biological effects of PQ^{2+} have been thoroughly investigated. This extremely toxic herbicide is a xenobiotic commonly used for generation of intracellular oxygen-derived active species and particularly superoxide radical. The toxicity of PQ^{2+} is usually attributed to intracellularly formed $PQ^{\bullet+}$, $O_2^{\bullet-}$, H_2O_2, and secondary ·OH radicals, which are generated through Fenton-like reactions (*1*, *2*) of metal ions bound to biological target (Biol-$M^{(n+1)+}$):

$$PQ \xrightarrow{\text{bioreduction}} PQ^{\bullet+} \quad (1)$$

$$O_2 + PQ^{\bullet+} \rightarrow O_2^{\bullet-} + PQ^{2+} \quad (2)$$

$$O_2^{\bullet-} + \text{Biol-}M^{(n+1)+} \rightarrow O_2 + \text{Biol-}M^{n+} \quad (3)$$

$$\text{Biol-}M^{n+} + H_2O_2 \rightarrow$$

$$H^+ + (\text{Biol-}M^{n}\text{-OOH})^{(n-1)+} \rightarrow \begin{cases} (\text{Biol-}M^{(n+1)+}\ {}^{\bullet}OH) + OH^- & (4a) \\ \text{Biol-}M^{(n+2)+} + 2OH^- & (4b) \end{cases}$$

Generally, attempts to block the cytotoxic effects of PQ^{2+} in experimental models included the use of catalase (*1*, *3*), superoxide dismutase (SOD) (*1*, *3*, *4*), chelating agents (*5*, *6*), redox-inactive metal ions (*7*), or inhibition of reductive enzyme systems (*8*). However, there is no effective medical treatment in cases of PQ^{2+} intoxication. A potential intervention is to employ cell-permeable stable nitroxide radicals, which scavenge xenobiotic-derived deleterious radicals (*9*), catalytically remove $O_2^{\bullet-}$ (*10*−*12*), and detoxify redox-active metal ions (*13*−*16*).

In the present study, the kinetics of PQ^{2+} and copper reactions with nitroxides were studied in the absence and presence of DNA using pulse radiolysis. The results imply that nitroxides would not be able to protect aerobic cells from PQ^{2+} toxicity. However, if PQ^{2+} induces toxicity also under anoxia, nitroxides would be effective protectants.

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Experimental Section

Chemicals. Paraquat (1,1′-dimethyl-4,4′-bipyridiniumdichloride), calf thymus DNA, type I, 2,9-dimethyl-1,10-phenanthroline (neocup), 1,10-phenanthroline (OP), 2,2′-bipyridyl (bipy), bathocupreine and cupric sulfate were obtained from Sigma; desferrioxamine (DFO) was a gift from Ciba Geigy. Xanthine oxidase (XO), NADH, and catalase were purchased from Boehringer Biochemicals. Diethylenetriaminopentaacetic acid (DTPA), TPO, and 4-OH-TPO were purchased from Aldrich. 4-Hydroxy-2,2,6,6-tetramethyl-*N*-hydroxypiperidine (4-OH-TPO-H) was obtained from Molecular Probes, Eugene, OR. All chemicals were prepared and used without further purification. Solutions were prepared with distilled water that had been further purified using a Milli-Q water purification system. A stock solution of DNA was prepared as ca. 1 mg/mL in 10 mM phosphate buffer at pH 7. The concentration of DNA per nucleotide was determined spectrophotometrically using $\epsilon_{260} = 6875\ M^{-1}\ cm^{-1}$.

Bacterial Cells. Bacterial cells included *Escherichia. coli* B SR-9, K12 parent strains AB 1157, KL-16, and KL16-N11, a mutant of KL16 with increased permeability to lipophilic compounds. The cells were grown at 37 °C in either LB or K growth medium supplemented with 0.5% glucose as a carbon source (*5*). Mid-log phase cells were washed twice by centrifugation at 15 °C (Wash Solution contained 0.85% NaCl, 2 mM phosphate, pH 7.4, and 1 mM $MgSO_4$), and resuspended at ~4 × 10^7 cells/mL in "Exposure Solution", which contained l mM $MgSO_4$ in 2 mM phosphate, 5 mM HEPES at pH 7.4 and 0.2% glucose. The cell suspensions were incubated for 10 min at 37 °C before the addition of PQ. The reaction was stopped after the samples were diluted 1:100 in a "Stop Solution" (Wash Solution supplemented with 200 μM DTPA, 0.3 mM histidine and 0.1% gelatin). Further dilutions were made as required, and aliquots were plated on LB-agar plates and incubated overnight at 37 °C.

Mammalian Cells. Chinese hamster V79 cells were grown in F12 medium supplemented with 10% fetal calf serum, penicillin, and streptomycin. Survival was determined by clonogenic assay, and the control plating efficiency ranged between 73 and 95%. Stock cultures of exponentially growing cells were trypsinized, rinsed, plated (5 × 10^5 cells/dish) in Petri dishes (60 or 100 mm), and incubated overnight at 37 °C prior to treatment. PQ^{2+} and other additives were added to exponentially growing cells in complete F12 medium for different periods of incubation. The cells were trypsinized, rinsed, counted, and plated in triplicate for macroscopic colony formation. Following appropriate incubation periods, colonies were fixed, stained, and last counted with the aid of a dissecting microscope.

Anoxic Experiments. The mammalian cells were plated into specially designed glass flasks sealed with soft rubber stoppers into which 19-gauge needles were pushed through to act as entrance and exit ports for an appropriate gas. The flask was also equipped with a ground glass sidearm vessel, which when rotated and inverted could deliver 0.2 mL of medium containing PQ. The flask was purged of O_2 at 37 °C for 45 min with pure N_2 mixed with 5% CO_2 (Matheson Gas Products), and then PQ^{2+} solution was added from the sidearm. For several experiments, sampling, dilutions and plating were performed using anoxic solutions, and the plates were incubated in anaerobic jars. A similar procedure was adopted for bacterial cells except that all cell suspensions were flushed with N_2 for 20 min. Gassing a suspension of actively metabolizing cells with N_2 completely purges the oxygen. If some trace of oxygen were left, the respiring cells would have consumed it. The removal of oxygen from the systems of mammalian cells was assured using Thermox probe (sensitivity of ca. 10 ppm). Furthermore, the appearance of the easily visible blue color of $PQ^{\bullet+}$ ($\epsilon_{605} = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$) with bacterial cells ensured strict anoxia, as $PQ^{\bullet+}$ reacts rapidly with O_2 (see below).

Electron Paramagnetic Resonance (EPR). Samples were drawn into a gas-permeable, 0.8 mm inner diameter, Teflon capillary. The capillary was inserted into a quartz tube and then placed within the EPR cavity. During the experiment, the sample within the spectrometer cavity was flushed with air or N_2, without disturbing the sample, and the EPR spectra were recorded on a Varian E9 X-band spectrometer operating at 9.45 GHz, 100 kHz modulation frequency, l G modulation amplitude, and 10−20 mW microwave power.

Pulse Radiolysis. Pulse radiolysis experiments were carried out with a Varian 7715 linear accelerator with 5-MeV electron pulses of 0.1−0.3 μs and 200 mA. A 200 W Xe−Hg lamp produced the analyzing light. Appropriate cutoff filters were used to minimize photochemistry. All measurements were made at room temperature in a 2-cm Spectrosil cell using three light passes (optical path length 6.2 cm).

Results

Kinetics. The kinetics of the reactions of $PQ^{\bullet+}$ with TPO, 4-OH-TPO, $Cu^{II}L_2$ (L = OP, bipy, neocup), and O_2 were studied at pH 7 using pulse radiolysis. In deaerated solutions cuprous ions disproportionate into $Cu^{2+}{}_{aq}$ and Cu°, and the solution becomes nonhomogeneous. Therefore, $Cu^{II}L_2$ (L = OP, bipy, neocup) were selected as model compounds, since the corresponding monovalent complexes are stable in deaerated solutions, and absorb highly in the visible region, i.e., $\epsilon_{435}(Cu(OP)_2{}^+) = 6770\ M^{-1}\ cm^{-1}$, $\epsilon_{430}(Cu(bipy)_2{}^+) = 4800\ M^{-1}\ cm^{-1}$, $\epsilon_{460}(Cu(neocup)_2{}^+) = 7200\ M^{-1}\ cm^{-1}$ (*17*). $PQ^{\bullet+}$ was instantaneously formed upon pulse-irradiation of N_2O-saturated solutions containing 0.1 M sodium formate, 1 mM PQ^{2+}, and 2 mM phosphate buffer (pH 7). Under these conditions, the following reactions take place (given in parentheses are the radiation-chemical yields of the species, defined as the number of species produced by 100 eV of energy absorbed):

$$H_2O \xrightarrow{\gamma} e^-{}_{aq}(2.6),\ {}^{\bullet}OH\ (2.7),\ H^{\bullet}\ (0.6),\ H_3O^+\ (2.6),\ H_2O_2\ (0.72) \quad (5)$$

$$e^-{}_{aq} + N_2O \rightarrow N_2 + OH^- + {}^{\bullet}OH \quad (6)$$

$${}^{\bullet}OH/H + HCO_2{}^- \rightarrow CO_2{}^{\bullet-} + H_2O/H_2 \quad (7)$$

$$CO_2{}^{\bullet-} + PQ^{2+} \rightarrow PQ^{\bullet+} + CO_2 \quad (8)$$

The decay of $PQ^{\bullet+}$ was followed at 605 nm in the presence of excess concentrations of all tested substrates and was found to obey first-order kinetics. Plots of k_{obs} were linearly dependent on the concentration of all substrates (Figure 1), and from the slopes of the lines, we determined $k_2 = (4.9 \pm 0.2) \times 10^8\ M^{-1}\ s^1$ and the rate constants for the reaction of $PQ^{\bullet+}$ with 4-OH-TPO, TPO, $Cu(OP)_2{}^{2+}$, $Cu(bipy)_2{}^{2+}$, and $Cu(neocup)_2{}^{2+}$ to be $(5.6 \pm 0.2) \times 10^4$, $(9.7 \pm 0.3) \times 10^3$, $(1.5 \pm 0.1) \times 10^9$, $(1.1 \pm 0.1) \times 10^9$, and $(1.9 \pm 0.1) \times 10^9\ M^{-1}\ s^1$, respectively.

EPR experiments showed that the reaction of $PQ^{\bullet+}$ with 4-OH-TPO yields the respective cyclic hydroxylamine (4-OH-TPO−H) as follows. 4-OH-TPO at 100 μM was incubated anoxically in 10 mM phosphate buffer (pH 7) containing 40 μM PQ, l mM NADH, and 0.1 units/mL of XO at room temperature. Under these conditions, in which NADH reduces PQ^{2+} enzymatically, the loss of 4-OH-TPO signal was monitored. The rate of the decay of the EPR signal was unaffected by the addition of 100 units/mL of SOD, and the signal was immediately

Table 1. Summary of the Rate Constants, Which Have Been Determined in the Present Study

k (M^{-1} s^{-1})	OP	bipy	neocup	
k_2				$(4.9 \pm 0.1) \times 10^8$
k_9				$(5.6 \pm 0.2) \times 10^4$
k_{10}				$(9.7 \pm 0.3) \times 10^3$
k_{11}	$(1.5 \pm 0.1) \times 10^9$	$(1.1 \pm 0.1) \times 10^9$	$(1.9 \pm 0.1) \times 10^9$	
k_{13}	$(4.1 \pm 0.2) \times 10^3$	$(8.8 \pm 0.1) \times 10^3$	nd[a]	
k_{-13}	179 ± 17	91 ± 10	nd	
k_{14}	$(2.2 \pm 0.1) \times 10^3$	nd	nd	
k_{-14}	68 ± 24	nd	nd	^

[a] nd, not determined.

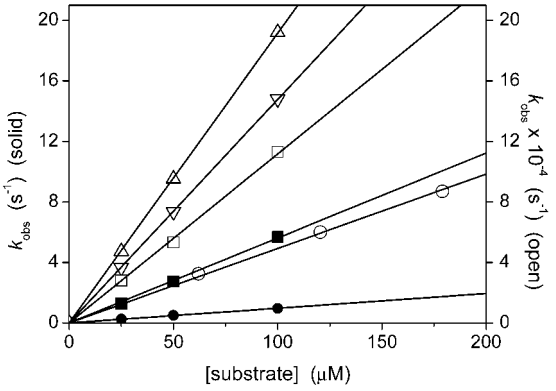

Figure 1. Dependence of the observed first-order rate constant for the decay of $PQ^{\bullet+}$ on the concentration of 4-OH-TPO (■), TPO (●), O_2 (○), $Cu(OP)_2^{2+}$ (▽), $Cu(bipy)_2^{2+}$ (□) and $Cu(neocup)_2^{2+}$ (△). All solutions where saturated with N_2O and contained 1 mM PQ, 0.1 M formate, and 2 mM phosphate buffer (pH 7). The dose rate was 5.7 Gy/pulse.

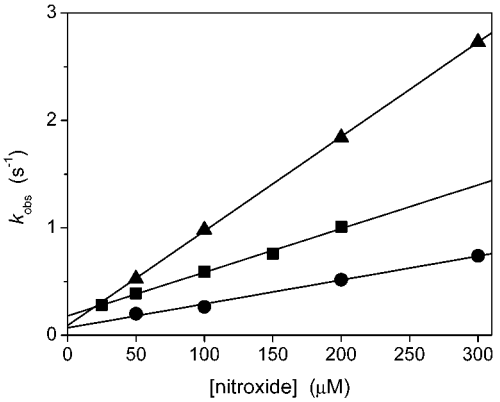

Figure 2. Dependence of the observed first-order rate constant for the decay of $Cu(OP)_2^+$ on [4-OH-TPO] (■) and [TPO] (●), and that of $Cu(bipy)_2^+$ on [4-OH-TPO] (▲). All solutions were N_2O-saturated, and contained 1 mM $Cu(OP)_2^{2+}$ [or $Cu(bipy)_2^{2+}$], 0.1 M formate and 2 mM phosphate buffer (pH 7). The dose rate was 5.7 Gy/pulse.

restored upon the addition of 1 mM ferricyanide. These results show the occurrence of reactions 9 and 10.

$$PQ^{\bullet+} + 4\text{-OH-TPO} \rightarrow PQ^{2+} + \text{4-OH-TPO-H} \qquad k_9 = (5.6 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1} \quad (9)$$

$$PQ^{\bullet+} + \text{TPO} \rightarrow PQ^{2+} + \text{TPO-H} \qquad k_{10} = (9.7 \pm 0.3) \times 10^3\ M^{-1}\ s^{-1} \quad (10)$$

In the case of $Cu^{II}L_2$, the decay of the absorption at 605 nm was accompanied by the build-up of the absorption in the visible region due to the formation of $Cu^{I}L_2$. We found that the yield of $Cu^{I}L_2$ was within experimental error identical to that of $PQ^{\bullet+}$, which indicates that $Cu^{II}L_2$ is reduced by $PQ^{\bullet+}$ to form $Cu^{I}L_2$ and PQ, i.e., reaction 11.

$$PQ^{\bullet+} + Cu^{II}L_2 \rightarrow PQ^{2+} + Cu^{I}L_2 \quad (11)$$

In another series of experiments, $Cu^{I}L_2$ was produced under the same conditions as described above for the generation of $PQ^{\bullet+}$. In this case, 1 mM $Cu^{II}L_2$ replaced PQ, and $Cu^{I}L_2$ was produced through reaction 12:

$$CO_2^{\bullet-} + Cu^{II}L_2 \rightarrow Cu^{I}L_2 + CO_2 \quad (12)$$

The decay of $Cu^{I}L_2$ (L = OP, bipy) in the presence of excess concentrations of 4-OH-TPO or TPO was followed and found to obey first-order kinetics. Plots of k_{obs} vs [nitroxide] resulted in straight lines with relatively large intercepts (Figure 2). $[Cu^{I}L_2]$ did not decay to zero and its yield at infinity time increased as [nitroxide] decreased. These results indicate that the reactions of $Cu^{I}L_2$ with nitroxides are in equilibrium, e.g., $k_{obs} = k_{14}[\text{TPO}] + k_{-14}[Cu^{II}L_2]$.

$$\text{4-OH-TPO} + Cu^{I}L_2 \overset{H^+}{\rightleftharpoons} \text{4-OH-TPO-H} + Cu^{II}L_2 \quad (13)$$

$$\text{TPO} + Cu^{I}L_2 \overset{H^+}{\rightleftharpoons} \text{TPO-H} + Cu^{II}L_2 \quad (14)$$

From the slopes and the intercepts of the lines in Figure 2, we obtained the rate constants for reactions 13 and 14, which are summarized in Table 1.

Effect of DNA on the Rates. The rate of reaction 11 was unaffected by DNA, whereas those of reactions 13 and 14 decreased as [DNA] increased. The results are summarized in Table 2.

Effect of 4-OH-TPO on Aerobic and Anoxic Toxicity of PQ. The cytocidal effect of PQ^{2+} on monolayered V79 cells in full medium under aerobic and anoxic conditions is demonstrated in Figure 3. The data were compiled from results obtained in several independent experiments conducted on different days, thus demonstrating the extent of experimental deviations. The killing process is characterized by a time lag, which is reflected by a shoulder in the respective survival curve. The loss of cell viability under anoxia shows a less pronounced shoulder compared to aerobic conditions, although the anoxic and aerobic rates of killing at the exponential phase hardly differ. Neither catalase (1000 units/mL) nor SOD (400 units/mL) protected against aerobic or anoxic toxicity of PQ, whereas 0.5 mM DFO, preincubated with the cells for 2 h before the addition of PQ, provided partial protection only in anoxia (data not shown). Accumulation of $PQ^{\bullet+}$ during anoxic experiments could not be observed,

Table 2. Effect of DNA on the Observed Rate Constants of Reactions 11, 13, and 14

	OP			bipy		neocup
[DNA] (μM)	$k_{obs}(11)$ (s^{-1})[a]	$k_{obs}(13)$ (s^{-1})[b]	$k_{obs}(14)$ (s^{-1})[c]	$k_{obs}(11)$ (s^{-1})[a]	$k_{obs}(13)$ (s^{-1})[b]	$k_{obs}(11)$ (s^{-1})[c]
0	$(3.7 \pm 0.3) \times 10^4$	1.01 ± 0.02	0.52 ± 0.02	$(2.8 \pm 0.2) \times 10^4$	1.83 ± 0.04	$(4.7 \pm 0.3) \times 10^4$
62.5		0.49 ± 0.03				
125		0.35 ± 0.02	0.24 ± 0.04			
150	$(3.4 \pm 0.2) \times 10^4$			$(2.9 \pm 0.3) \times 10^4$		
250		0.18 ± 0.03	0.14 ± 0.01		1.08 ± 0.03	
300	$(3.7 \pm 0.2) \times 10^4$					$(3.4 \pm 0.3) \times 10^4$

[a] Reaction of $PQ^{\bullet+}$ with 25 μM $Cu^{II}L_2$. [b] Reaction of $Cu^{I}L_2$ with 200 μM 4-OH-TPO. [c] Reaction of $Cu^{I}L_2$ with 200 μM TPO.

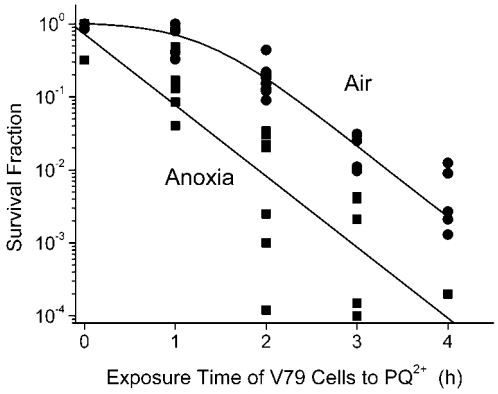

Figure 3. Aerobic and anoxic toxic effects of PQ^{2+} on cultured mammalian cells. Monolayered Chinese hamster V79 cells at $\sim 5 \times 10^5$ cells/dish were incubated with 10 mM PQ^{2+} either under aerobic conditions (circles) or under nitrogen (squares). At various time points, the cells were trypsinized, counted, plated in triplicates and incubated for 7 days for macroscopic determination of colony forming ability. The results presented were pooled from seven experiments performed on several days.

because the cells reduce PQ^{2+} slowly, and the steady-state concentration of $PQ^{\bullet+}$ was below detection. Preincubation of V79 cells under air and anoxia with buthionine sulfoximine, which inhibits GSH synthesis, increased the rate of their killing (data not shown). This agrees with previous reports that lowering the level of cellular GSH renders the cells more susceptible to PQ^{2+} (*18*, *19*).

In contrast to mammalian cells, shorter exposure times and lower [PQ] were needed to inflict significant killing of bacterial cells, provided $CuSO_4$ was added. Figure 4 compares typical anoxic and aerobic survival curves. Control experiments showed that 1 mM PQ^{2+} alone was not toxic both under air and anoxia, whereas $CuSO_4$ alone at 0.5−1.5 μM induced some bacterial cell killing only under anoxia, but far less than that observed when PQ^{2+} was also present (Figure 4). When the cells were incubated with 1 mM PQ^{2+} alone or together with 1.5 μM $CuSO_4$, the blue color of $PQ^{\bullet+}$ was progressively accumulated, which ensured the absence of O_2. The accumulated $PQ^{\bullet+}$ immediately and fully disappeared upon anoxic addition of 2 mM 4-OH-TPO or 50 μM $CuSO_4$ or exposure to air, indicating that PQ, which is reduced by the cells, can be regenerated through the oxidation of $PQ^{\bullet+}$ by 4-OH-TPO or Cu^{II} or O_2. The rate of PQ-induced cell killing was sensitive to the metabolic state of the cells and their energy source and varied considerably among various experiments. Addition of 200 μM of DTPA, neocup, or bathocuproine fully inhibited cell killing. Adding $CuSO_4$ following 10 min preincubation of the cells with PQ^{2+} alone resulted in survival curves without a shoulder.

4-OH-TPO had hardly any effect on the aerobic toxicity PQ^{2+} toward mammalian and bacterial cells. In contrast,

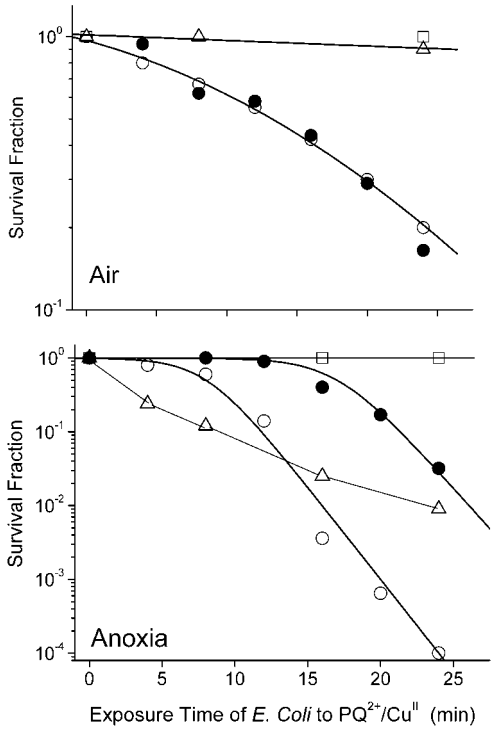

Figure 4. Toxicity of PQ^{2+} and Cu^{II} to *E. coli*, and the effect of 4-OH-TPO on PQ^{2+}/Cu^{II} toxicity under air and anoxia. Washed *E. coli* KL16-N11 were incubated at 37 °C in buffer ($\sim 4 \times 10^7$ cells/mL) containing 0.2% glucose with 1 mM PQ^{2+} (open squares), 1.5 μM $CuSO_4$ (open triangles), 1 mM PQ^{2+} and 1.5 μM $CuSO_4$ (open circles) or 1 mM PQ^{2+} and 1.5 μM $CuSO_4$ and 5 mM 4-OH-TPO (solid circles). Top: under air. Bottom: under anoxia.

it provided significant protection to all *E. coli* cells incubated with $PQ/CuSO_4$ under anoxia (Figure 4). The nitroxide provided some protection to anoxic mammalian cells, but the effect was hardly significant and less pronounced compared to *E. coli*. The hydroxylamine, 4-OH-TPO-H, did not affect the anoxic and aerobic toxicity of PQ^{2+} toward all cells.

It was difficult to exclude the possibility that PQ-induced cellular damage under anoxia actually takes place during plating and aerobic colony growth. That is, O_2 might be required for damage during the growth of the treated cells rather than during their initial exposure to PQ. To examine this possibility, *E. coli* KL16-N11 cells were exposed anoxically to 1 mM PQ^{2+} and 1.25 μM $CuSO_4$ and later sampled, diluted, plated on glucose containing plates, and incubated overnight either aerobically or anoxically. The respective survival curves obtained are displayed in Figure 5. The results indicate that the actual damage took place during the anoxic exposure to $PQ/CuSO_4$. This conclusion is corroborated by results displayed by the survival curve in Figure 5, which

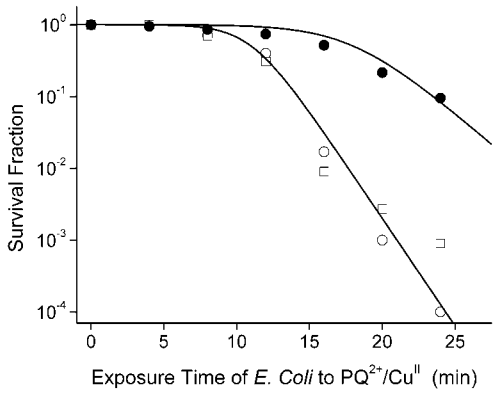

Figure 5. Effect of oxygen on survival during plating and growth following exposure of anoxic *E. coli* to PQ^{2+}/Cu^{II}. Time dependence of cell survival following anoxic incubation of *E. coli* KL16-N11 at ~4 × 10^7 cells/mL with 1 mM PQ^{2+} and l.25 μM $CuSO_4$ at 37 °C in the absence (open symbols) and the presence (solid symbols) of 5 mM 4-OH-TPO. At various time points the cells were sampled into "Stop Solution", diluted, plated, and incubated overnight at 37 °C either under air (circles) or under anoxia (squares).

describes the effect of 4-OH-TPO on cells that have been treated with $PQ/CuSO_4$ under anoxia.

Discussion

The commonly accepted mechanism for PQ^{2+} toxicity in aerobic conditions, i.e., reactions 1−4, implies that nitroxides would protect the cells from the aerobic toxicity of PQ^{2+} by competing with O_2 for $PQ^{\bullet+}$ and/or with H_2O_2 for the reduced metal complex. However, the kinetics results show that the rate of the reaction of $PQ^{\bullet+}$ with O_2 exceeds ca. 4 orders of magnitude those with TPO and 4-OH-TPO (Figure 1). Hence, even 10 mM nitroxides cannot compete efficiently with O_2 for $PQ^{\bullet+}$ in aerated systems, i.e., $k_2 \times [O_2] > k_9 \times$ [4-OH-TPO-H] (or $k_{10} \times$ [TPO]). Furthermore, our results show that the rates of reactions 13 and 14 decrease as [DNA] increases (Table 1), which suggests that nitroxides react significantly slower with reduced metals that are bound to biological targets. In contrast, DNA had previously been shown to have no effect on reaction 4 (*17*). Therefore, nitroxides most probably will not compete efficiently with H_2O_2 for the reduced metal bound to a biological molecule, i.e., $k_4 \times [H_2O_2] > k_{13} \times$ [4-OH-TPO] (or $k_{14} \times$ [TPO]). We conclude that the results of the kinetics experiments provide a reasonable explanation for the failure of 4-OH-TPO to protect aerobic bacterial and mammalian cells from PQ^{2+} toxicity.

The rate of the reaction of $PQ^{\bullet+}$ with $Cu^{II}L_2$ was found to be unaffected by DNA (Table 1). Furthermore, copper toxicity has been attributed mainly to Cu^{I} and was observed predominantly for cells subjected to anoxic conditions (*20*−*22*). Cu^{I}, though not Cu^{II}, was also found to sensitize mammalian cells to ionizing radiation (*23*). Therefore, it is anticipated that nitroxides would be effective protectants if PQ^{2+} induces toxicity also under anoxia. Surprisingly, we found that PQ^{2+}/Cu^{II} toxicity under anoxia was even greater than that under air (Figures 3 and 4), and 4-OH-TPO protected the cells only under anoxia (Figure 4). In the absence of metal ions, $PQ^{\bullet+}$ is practically not toxic both under aerobic and anoxic conditions (Figure 4). However, $PQ^{\bullet+}$ accumulated also in the presence of 0.5−1.5 μM $CuSO_4$, where the cells were rapidly killed. Thus, excluding any role played under anoxia by $PQ^{\bullet+}$, $HO_2^{\bullet}/O_2^{\bullet-}$, H_2O_2 or $^{\bullet}OH$, the present observations implicate reduced metal as a prime deleterious species. Preincubation of the cells with PQ^{2+} alone prior to the addition of $CuSO_4$ allowed accumulation of $PQ^{\bullet+}$ and yielded survival curve without a shoulder supporting the former conclusion. Therefore, nitroxides can potentially protect the cells from PQ^{2+} toxicity either by competing with Cu(II) for $PQ^{\bullet+}$ or by oxidizing Cu^{I}. The effects of Cu^{II} and nitroxides on the rate of the accumulation of $PQ^{\bullet+}$ and the mechanism underlying the anoxic toxicity of PQ^{2+} are currently under investigation.

We note that Fridovich et al. (*5*, *24*) did not observe toxicity upon plating PQ-treated *E. coli* cells under anoxia. The apparent contradiction of the present observation with previous reports can be reconciled by a close examination of the bacterial strains used, respective growth conditions of the cells, their metabolic state, and conditions of exposure to PQ. Presumably, adventitious traces of transition metal ions, which were not deliberately removed from the reaction mixture, contributed to cell injury. The killing process was terminated using a solution of high osmolarity, whereas, cells plating and colony growth took place under either air or anoxia. Under such conditions, the presence of oxygen during colony growth of *E. coli B* was found essential for killing. In the present study *E. coli B* and *K12* cells were washed thoroughly at room temperature and incubated in buffer with PQ^{2+} in the presence of an energy source and a subtoxic concentration of $CuSO_4$ at 37 °C to maintain the reductive capacity of the cells.

In Conclusion. The present study shows that PQ^{2+} is toxic to bacteria and mammalian cells in the absence of oxygen, the toxicity under anoxia exceeds that under air, and nitroxides provide significant protection only under anoxia. Bacterial cells, which have higher reductive capacity than mammalian cells, reduce PQ^{2+} much more rapidly and are more susceptible to both its aerobic and anoxic toxicity. These results suggest, though not prove, that under anoxic conditions the reduced metal, i.e., Cu(I), is the toxic species, and PQ^{2+} facilitates the reduction of the metal.

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