Possible Role of Bacterial Siderophores in Inflammation

Iron Bound to the *Pseudomonas* Siderophore Pyochelin Can Function as a Hydroxyl Radical Catalyst

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Abstract

Tissue injury has been linked to neutrophil associated hydroxyl radical (· OH) generation, a process that requires an exogenous transition metal catalyst such as iron. In vivo most iron is bound in a noncatalytic form. To obtain iron required for growth, many bacteria secrete iron chelators (siderophores). Since *Pseudomonas aeruginosa* infections are associated with considerable tissue destruction, we examined whether iron bound to the *Pseudomonas* siderophores pyochelin (PCH) and pyoverdin (PVD) could act as · OH catalysts. Purified PCH and PVD were iron loaded (Fe-PCH, Fe-PVD) and added to a hypoxanthine/xanthine oxidase superoxide- ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2)-generating system. Evidence for · OH generation was then sought using two different spin-trapping agents (5,5 dimethyl-pyrroline-1-oxide or N-t-butyl-α-phenylnitrone), as well as the deoxyribose oxidation assay. Regardless of methodology, · OH generation was detected in the presence of Fe-PCH but not Fe-PVD. Inhibition of the process by catalase and/or SOD suggested · OH formation with Fe-PCH occurred via the Haber–Weiss reaction. Similar results were obtained when stimulated neutrophils were used as the source of $\cdot O_2^-$ and H_2O_2. Addition of Fe-PCH but not Fe-PVD to stimulated neutrophils yielded · OH as detected by the above assay systems. Since PCH and PVD bind ferric (Fe^{3+}) but not ferrous (Fe^{2+}) iron, · OH catalysis with Fe-PCH would likely involve $\cdot O_2^-$-mediated reduction of Fe^{3+} to Fe^{2+} with subsequent release of "free" Fe^{2+}. This was confirmed by measuring formation of the Fe^{2+}-ferrozine complex after exposure of Fe-PCH, but not Fe-PVD, to enzymatically generated $\cdot O_2^-$. These data show that Fe-PCH, but not Fe-PVD, is capable of catalyzing generation of · OH. Such a process could represent as yet another mechanism of tissue injury at sites of infection with *P. aeruginosa*. (*J. Clin. Invest.* 1990. 86:1030–1037.) **Key words: pyoverdin • neutrophil • cystic fibrosis • lung injury • hydroxyl radical**

Introduction

Neutrophil-derived oxidants have been suggested as important contributors to host injury in a wide array of inflammatory states (1). In vitro, superoxide anion ($\cdot O_2^-$) and hydrogen peroxide (H_2O_2), generated by the neutrophil "respiratory burst" can react with an exogenous iron catalyst to form hydroxyl radical (· OH) via the Haber–Weiss reaction shown below (2, 3).

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow \cdot OH + OH^- + Fe^{3+}$$

$$\overline{\hspace{6cm}}$$

$$H_2O_2 + \cdot O_2^- \rightarrow \cdot OH + OH^- + O_2$$

Hydroxyl radical is a highly reactive oxidant. Several lines of evidence point to · OH as an important mediator of acute lung injury and other forms of phagocyte-associated tissue damage (1, 4, 5–7). Although it has been reported that neutrophils have the endogenous capacity for · OH formation (8–13), the experimental techniques used in these studies have been criticized for a lack of specificity (14). Recent studies using spin trapping and other techniques have strongly suggested that an exogenous transition metal catalyst must be present for neutrophil activation to result in · OH generation (3, 15–20).

In humans "free" iron is almost nonexistent, present at a level of $\sim 10^{-18}$ M (21). Most iron, whether intra- or extracellular, is bound to proteins or incorporated into other molecules. Iron bound to either of the two principal extracellular iron chelates, transferrin and lactoferrin, is not catalytic for the Haber–Weiss reaction (22–25). Although recent evidence suggests that iron bound to ferritin or hemoglobin may be able to act as a · OH catalyst (26–28), access of phagocyte-derived $\cdot O_2^-/H_2O_2$ to these intracellular iron complexes is limited by cellular antioxidant systems (1).

Iron is an essential nutrient for microbial growth and metabolism for which invading microorganisms must compete with host iron-binding proteins (29, 30). Host sequestration of iron has been suggested as an important means of defense from bacterial pathogens (21, 31, 32). Under iron-limited conditions many bacteria and fungi secrete low-molecular weight compounds with high iron-binding affinities known as siderophores (29, 30, 33, 34). These siderophores can abstract iron from some host sources, making it available for uptake and utilization by the microorganism (29, 30, 35, 36).

Pseudomonas aeruginosa, a bacterial pathogen associated with severe necrotizing pneumonia in compromised hosts as well as progressive pulmonary deterioration in cystic fibrosis

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(37), secretes two siderophores; pyochelin (PCH)[1] and pyoverdin (PVD) (33, 34). Indirect evidence has been obtained that siderophore generation occurs in vivo at sites of *P. aeruginosa* infection (38) where neutrophil-derived $\cdot O_2^-$ and H_2O_2 would also be present. Accordingly, we assessed whether iron bound to PCH or PVD could catalyze $\cdot OH$ formation via the Haber–Weiss reaction since the generation of $\cdot OH$ from the interaction of stimulated phagocytes and siderophore-bound iron could contribute to the extensive tissue injury characteristic of pseudomonas infection.

Methods

Reagents. Diethylenetriaminepentaacetic acid (DTPA), SOD, PMA, DMSO, hypoxanthine, bovine serum albumin, dihydrocytochalasin B, zymosan A, catalase, *N*-t-butyl-α-phenyl-nitrone (PBN), 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2,4 triazine (ferrozine), 2-deoxyribose, TCA, 2,9-dimethyl-1,10 phenanthroline (neocuproine) and thiobarbituric acid (TBA) were purchased from Sigma Chemical (St. Louis, MO). Xanthine oxidase was purchased from Sigma Chemical Co. or Boehringer Mannheim Biochemicals (Indianapolis, IN). Results were not altered by the source of xanthine oxidase. Ferrous ammonium sulfate, ferric chloride, and H_2O_2 were purchased from Fischer Scientific (Fairlawn, NJ). Zymosan was opsonized (OZ) by incubation in 100% normal pooled human serum (37°C for 30 min) as previously described (3).

Siderophore preparation. Pyochelin (PCH) and pyoverdin (PVD) were purified from *P. aeruginosa* broth culture as previously described (33, 34). Briefly, *P. aeruginosa* strain PA01 (ATCC 15692; American Type Culture Collection, Rockville, MD) was grown to log phase under iron-depleted conditions. PCH secreted into the media was extracted with dichloromethane and 1% acetic acid followed by purification by TLC. PVD was purified from a separate broth culture by a series of filtration/precipitation steps culminating in gel IEF. PCH was suspended in DMSO and PVD suspended in H_2O.

To iron load either PVD or PCH, sufficient $FeCl_3$ was added at pH 5.0 to achieve 50% saturation based on the known molar binding ratio of Fe/PCH (1:2) and Fe/PVD (1:1). 50% saturated PCH (Fe-PCH) and PVD (Fe-PVD) was chosen for study to eliminate the possible contribution of "free" iron resulting from inadvertent overloading of the molecules.

Neutrophil separation. Neutrophils were separated from venous blood of normal human volunteers using dextran sedimentation and a Ficoll-Hypaque gradient according to the method of Borregaard et al. (39). Neutrophils were then maintained on ice in HBSS without phenol red (University of Iowa Cell Culture Facility, Iowa City, IA) until usage.

Spin trapping. Electron spin resonance (ESR) detection of spin adducts was performed using a spectrometer (model E104 A ESR; Varian Associates, Palo Alto, CA). Desired reaction mixtures (0.5 ml) were prepared in glass tubes and transferred to a quartz ESR flat cell, which was in turn placed in the cavity of the ESR spectrometer. Sequential ESR scans were then obtained at 25°C. Unless otherwise noted ESR spectrometer settings were: microwave power, 20 mW; modulation frequency 100 kHz; modulation amplitude, 1.0 G; and response time, 1 s. Other settings are noted in the figure legends.

Deoxyribose oxidation. Deoxyribose oxidation was performed using a slight modification of the methods of Greenwald et al. (18). Briefly, 100 μM Fe-PVD or 50–200 μM Fe-PCH was added to buffer (H_2O or HBSS) containing deoxyribose (5 mM), and in some cases 5 × 10^6 neutrophils or 200 μM hypoxanthine to a final volume of 1 ml. After the addition of 200 μM H_2O_2, 100 ng/ml of the neutrophil stimulant PMA, or 0.06 U/ml xanthine oxidase, respectively, to initiate $\cdot O_2^-/H_2O_2$ generation reaction mixtures were incubated 15–30 min at 37°C. Reactions were terminated by addition of 1.0 ml TCA (6%) and 0.5 ml TBA (1% wt/vol in 0.5 M NaOH), after which cells (if present) were pelleted (12,400 *g*, 5 min). The supernatant was transferred to glass tubes, boiled for 15 min, and A_{532} of each mixture determined using a spectrophotometer (model DU-30; Beckman Instruments, Inc., Palo Alto, CA). 500 Units/ml catalase or 30 U/ml SOD were included in the original reaction mixture in some experiments.

Iron release from siderophores. Ferrozine/Fe^{2+} complex formation as measured by A_{562} was used to assess $\cdot O_2^-$-mediated reduction and subsequent release of siderophore bound Fe^{3+} (40). Mixtures containing 10 mM ferrozine, 200 μM hypoxanthine, 0.06 U/ml xanthine oxidase and siderophore (PVD, 100 μM, PCH, 50–100 μM) were assayed for an increase in A_{562}. 30 U/ml SOD was included in some experiments to confirm that iron release was dependent on the generation of $\cdot O_2^-$. Results were identical regardless of whether or not neocuproine was included in the reaction mixture to prevent formation of a copper-ferrozine complex. In some experiments 500 U/ml catalase was included to prevent the reoxidation of Fe^{2+} by H_2O_2. Although this resulted in an increased rate of Fe^{2+}–ferrozine complex formation, it had no effect on total Fe^{2+} release observed.

Results

The reaction of xanthine oxidase with xanthine or hypoxanthine results in the generation of $\cdot O_2^-$ and H_2O_2 but not $\cdot OH$. Detection of $\cdot OH$ after the addition of an iron chelate to a mixture of (hypo)xanthine and xanthine oxidase has been routinely used to assess the capacity of that chelate to catalyze the Haber–Weiss reaction (22–24). Accordingly, 50% iron-saturated preparations of pyochelin (Fe-PCH) or pyoverdin (Fe-PVD) were added to a hypoxanthine/xanthine oxidase system and evidence for $\cdot OH$ formation sought using a previously described spin-trapping system consisting of 5,5, dimethyl-1-pyrroline-1-oxide (DMPO) and DMSO (3, 19, 20).

In the absence of DMSO, DMPO reacts with $\cdot O_2^-$ and $\cdot OH$ to yield 2,2, dimethyl-5-hydroperoxy-1-pyrridinyloxyl (DMPO/$\cdot$OOH) and 2,2 dimethyl-5-hydroxy-1-pyrridinyloxyl (DMPO/$\cdot$OH) (41, 42). However, DMPO/$\cdot$OH may also arise from the decomposition of DMPO/$\cdot$OOH, rendering DMPO/$\cdot$OH detection unreliable as evidence for the presence of $\cdot OH$. DMSO reacts with $\cdot OH$ to yield methyl radical ($\cdot CH_3$), which can be spin trapped by DMPO as 2,2,5-trimethyl-1-pyrridinyloxyl (DMPO/$\cdot CH_3$). When the concentration of DMSO exceeds DMPO, as routinely is the case in our system, the presence of $\cdot OH$ is manifested as DMPO/$\cdot CH_3$ (3). Since DMPO/$\cdot CH_3$ is not a direct decomposition product of DMPO/$\cdot$OOH its detection provides more specific spin-trap evidence of $\cdot OH$ generation (43).

Consistent with previous work (20), ESR spectra obtained during the reaction in H_2O of xanthine oxidase and hypoxanthine in the presence of DMPO, DMSO, and DTPA was composed primarily of DMPO/$\cdot$OOH and DMPO/$\cdot$OH (Figs. 1 *A* and 2 *A*). The small DMPO/$\cdot CH_3$ peaks present were inhibited by the inclusion of SOD but not catalase (not shown), indicating they arose from spin trapping the small amount of $\cdot OH$ that may arise from DMPO/$\cdot$OOH breakdown (3, 43).

A marked increase in DMPO/$\cdot CH_3$ was detected when Fe-PCH (Fig. 1 *B*), but not Fe-PVD (Fig. 2 *B*), was added to the hypoxanthine/xanthine oxidase system. The presence of

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[1] *Abbreviations used in this paper:* DMPO, 5,5-dimethyl-pyrroline-1-oxide; DTPA, diethylenetriaminepentaacetic acid; ESR, electronic spin resonance; OZ, opsonized zymosan; PBN, *N*-t-α-phenyl-nitrone; PCH, pyochelin; PVD, pyoverdin; TBA, thiobarbituric acid.

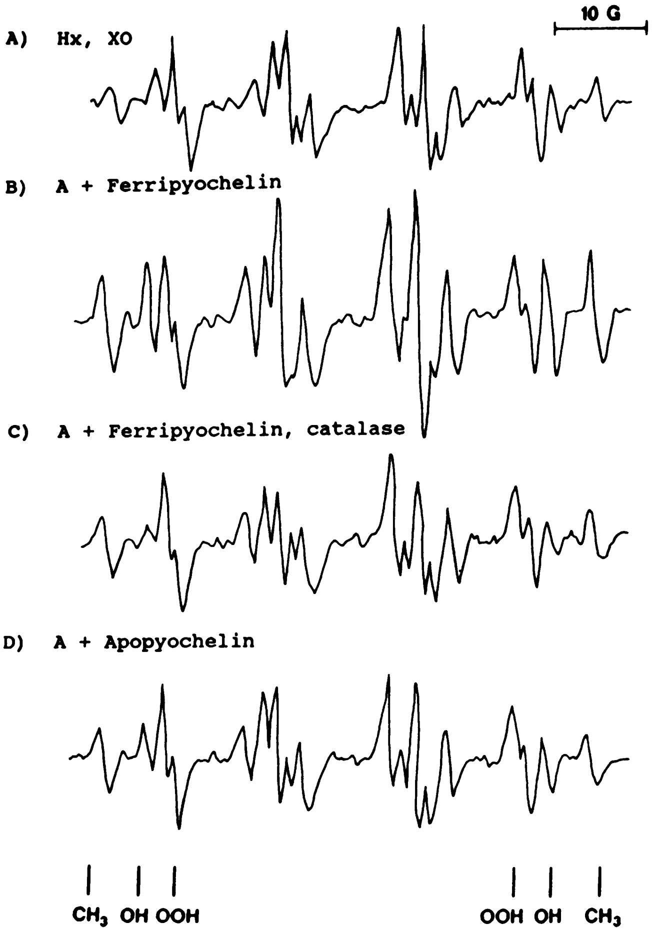

Figure 1. Representative ESR spectra (n = 3–5) after the addition of 0.06 U/ml xanthine oxidase to a solution containing: (*A*) DMSO (140 mM), DMPO (100 mM), DTPA (0.1 mM), hypoxanthine (HX, 0.2 mM); (*B*) contents of *A* plus Fe-PCH (0.2 mM); (*C*) contents of *A* plus Fe-PCH (0.2 mM) and catalase (500 U/ml); and (*D*) contents of *A* plus apopyochelin. Location of high- and low-field peaks corresponding to DMPO/$\cdot CH_3$, DMPO/$\cdot$OH, and DMPO/$\cdot$OOH are indicated as *CH_3, OH,* and *OOH,* respectively. Receiver gain was 3.2 × 10^4 and sweeprate 12.5 G/min.

catalase returned the DMPO/$\cdot CH_3$ peak amplitude to that observed in the absence of Fe-PCH (Fig. 1 *C*) as would be expected if the DMPO/$\cdot CH_3$ increase resulted from spin trapping of $\cdot$OH formed via the Haber–Weiss reaction. No spectra were observed with the omission of xanthine oxidase (not shown). Substitution of apopyochelin for Fe-PCH (Fig. 1 *D*) yielded only background DMPO/$\cdot CH_3$ peak amplitudes. These data suggest that Fe-PCH but not Fe-PVD is capable of catalyzing the Haber–Weiss reaction.

It has been reported that the stability of DMPO/$\cdot CH_3$ is decreased in the presence of $\cdot O_2^-$ suggesting that failure to detect DMPO/$\cdot CH_3$ may not be an absolute indicator of the lack of $\cdot$OH formation (19, 44, 45). Recently we developed a new means of spin trapping $\cdot$OH using DMSO and the spin trap PBN (46). In the presence of DMSO and PBN, the generation of $\cdot$OH in aerated solutions yields a single stable nitroxide species, which we have assigned to PBN/$\cdot OCH_3$ (46). The use of PBN in place of DMPO offers two advantages when investigating $\cdot$OH generation from $\cdot O_2^-$ and H_2O_2. First, unlike DMPO/$\cdot CH_3$, PBN/$\cdot OCH_3$ appears to be resistant to $\cdot O_2^-$-induced degradation (46). Second, reaction of $\cdot O_2^-$ with PBN does not yield a stable spin adduct.

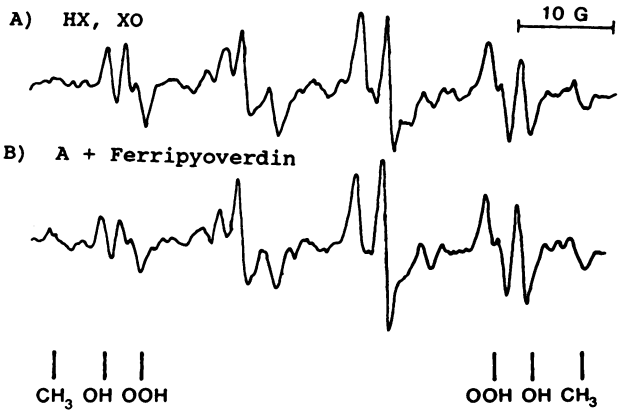

Figure 2. ESR spectra representative of 4 separate experiments obtained after the addition of xanthine oxidase (0.06 U/ml) to a solution containing: (*A*) DMSO (140 mM), DMPO (100 mM), DTPA (0.1 mM), and hypoxanthine (HX, 0.2 mM); and (*B*) contents of *A* plus Fe-PVD (0.1 mM). Location of high- and low-field peaks corresponding to DMPO/$\cdot CH_3$, DMPO/$\cdot$OH, and DMPO/$\cdot$OOH are indicated as *CH_3, OH,* and *OOH,* respectively. ESR spectrometer settings were as in Fig. 1.

Using this PBN/DMSO spin trapping system we reassessed the potential for Fe-PCH and Fe-PVD to act as $\cdot$OH catalysts. The results are seen in Fig. 3. Consistent with the DMPO results, $\cdot$OH production (PBN/$\cdot OCH_3$) was detected in solutions containing hypoxanthine, xanthine oxidase, and Fe-PCH (Fig. 3 *B*) but not Fe-PVD (Fig. 3 *D*). Catalase inhibited PBN/$\cdot OCH_3$ spin adduct formation (Fig. 3 *C*) consistent with Haber-Weiss mediated $\cdot$OH formation.

These spin trapping data (Figs. 1–3) provided strong evidence for the ability of Fe-PCH but not Fe-PVD to act as a Haber–Weiss catalyst. However, to further confirm these results we used an alternative $\cdot$OH detection system, the deoxyribose oxidation assay. In the presence of $\cdot$OH, 2-deoxyribose is oxidized to yield a compound which when exposed to thiobarbituric acid and boiled forms a chromogen with an absorbance maximum of 532 nm (A_{532}) (18, 47). The magnitude of

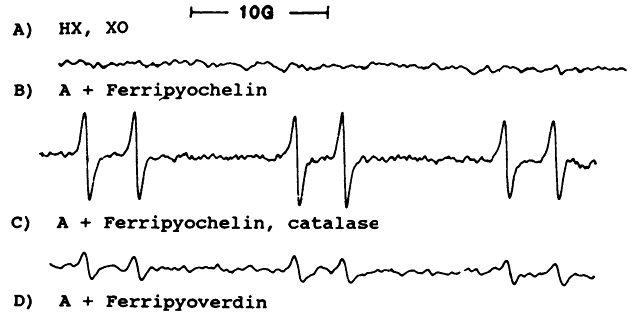

Figure 3. ESR spectra representative of three to five separate experiments obtained after the addition of xanthine oxidase (0.06 U/ml) to solutions containing: (*A*) DMSO (140 mM), PBN (10 mM), DTPA (0.1 mM), and hypoxanthine (*HX,* 0.2 mM); (*B*) contents of *A* plus Fe-PCH (0.2 mM); (*C*) contents of *A* plus Fe-PCH (0.2 mM) and catalase (500 U/ml); and (*D*) contents of *A* plus Fe-PVD (0.1 mM). The species detected in *B* is that of PBN/$\cdot OCH_3$. Receiver gain was 5 × 10^4 and sweeprate 10 G/min.

TBA-reactive deoxyribose oxidation products formed correlates with the amount of $\cdot OH$ generated. Detection of such products appears to be a sensitive and relatively specific assay for the generation of $\cdot OH$. Analogous to results with the known $\cdot OH$ catalyst Fe-EDTA, addition of xanthine oxidase to a solution of hypoxanthine, 2-deoxyribose and Fe-PCH resulted in SOD- and catalase-suppressible chromogen formation (Table I). This was not observed with Fe-PVD (Table I) confirming our spin-trapping results. Variation in background chromogen formation observed in reactions containing hypoxanthine, 2-deoxyribose, and xanthine oxidase but no exogenous iron likely resulted from the presence of trace iron contamination of buffer and enzyme preparations (48).

It has been suggested that the reaction of H_2O_2 with some ferric iron chelates may yield a hydroxyl radical–like species in the absence of an exogenous source of $\cdot O_2^-$ (49–51). To determine whether Fe-PCH was capable of participating in such a reaction, formation of TBA-reactive deoxyribose oxidation products was determined after the addition of 200 μM H_2O_2 to different iron chelates (50 μM)-Fe-PCH, Fe^{3+}-EDTA, and Fe^{2+}-EDTA (Table I). In the absence of exogenous iron no evidence of $\cdot OH$ was detectable. Fe-PCH and Fe^{3+}-EDTA resulted in generation of a species that oxidized deoxyribose to a similar extent. The magnitude of this deoxyribose oxidation was considerably less than that observed with Fe^{2+}-EDTA.

To further confirm that chromagen formation in the deoxyribose assay resulted from the presence of $\cdot OH$ we attempted to assess the impact on chromagen formation of various scavengers that have been shown to have differing reaction rates for $\cdot OH$ vs. other oxidants (52). These studies were confounded by the fact that Fe-PCH was suspended in DMSO, a potent $\cdot OH$ scavenger (52). Other solvents were not suitable, either because of volatility or inherent scavenger activity.

Table I. Ability of Pyochelin and Pyoverdin to Catalyze Hydroxyl Radical Formation by Hypoxanthine/Xanthine Oxidase or Hydrogen Peroxide

Ferripyochelin	A_{532}	Ferripyoverdin	A_{532}
HX/XO	0.068	HX/XO	0.000
HX/XO + Fe^{3+}-EDTA	0.390	HX/XO + Fe^{3+}-EDTA	0.600
HX/XO + Fe^{3+}-EDTA + catalase	0.052	HX/XO + Ferripyoverdin	0.005
HX/XO + Fe^{3+}-EDTA + SOD	0.092		
HX/XO + ferripyochelin	0.210		
HX/XO + ferripyochelin + catalase	0.055		
HX/XO + ferripyochelin + SOD	0.075		
HX/XO + apopyochelin	0.072		
H_2O_2	0.022		
H_2O_2 + Fe^{3+}-EDTA	0.174		
H_2O_2 + Fe^{2+}-EDTA	0.694		
H_2O_2 + ferripyochelin	0.165		

Formation of TBA-reactive deoxyribose oxidation products measured as A_{532} representative of 3–10 separate experiments after the addition of various iron chelates to H_2O_2 or the reaction of hypoxanthine (*HX*) and xanthine oxidase (*XO*). Background activity with H_2O_2 or HX/XO in the absence of exogenous iron is related to iron contaminants in buffer and/or enzyme preparations.

At sites of infection human phagocytes, particularly neutrophils, would be the likely source of $\cdot O_2^-$ and H_2O_2 that could interact with bacterial siderophores to form $\cdot OH$. In addition to inducing $\cdot O_2^-$ formation, neutrophil stimulation also results in extracellular release of a variety of enzymes and other compounds from cytoplasmic storage granules (53). Previous studies have demonstrated that the neutrophil granule components lactoferrin and myeloperoxidase diminish $\cdot OH$ formation by neutrophils supplemented with catalytic iron by chelating iron in a noncatalytic form and scavenging H_2O_2, respectively (15, 20, 54). Among the enzymes secreted by stimulated neutrophils are a variety of proteases that, while unlikely to impact on $\cdot OH$ generation directly, could alter the structure and thereby the iron-binding characteristics of either PCH or PVD. Such changes could lead to either loss or gain, of Haber–Weiss catalytic properties. Thus the ability or inability of pseudomonas siderophores to catalyze formation of $\cdot OH$ by the hypoxanthine/xanthine oxidase system by no means assures similar results when stimulated neutrophils are the source of $\cdot O_2^-$ and H_2O_2.

To determine what impact stimulated neutrophils had on the catalytic potential of Fe-PCH or Fe-PVD, spin-trap evidence of $\cdot OH$ was sought after PMA stimulation of human neutrophils in the presence of Fe-PCH or Fe-PVD. Consistent with earlier work, PMA stimulation of neutrophils in the presence of DMPO, DMSO, and DTPA, but without exogenous iron, yielded only $\cdot O_2^-$-derived DMPO spin adducts (Fig. 4 *A*). Iron supplementation resulted in catalase suppressible DMPO/$\cdot CH_3$, (Fig. 4, *B* and *C*). ESR spectra of solutions containing PMA stimulated neutrophils, DMPO, DTPA, DMSO, and Fe-PVD yielded no evidence of $\cdot OH$ (catalase inhibitable DMPO/$\cdot CH_3$) formation (Fig. 4 *D*).

Unfortunately, when Fe-PCH was used as the iron source in buffers necessary to maintain neutrophil viability, nitroxide artifacts similar to those described in other systems (55) were encountered which prevented accurate interpretation of the results (not shown). Fortunately, substitution of PBN for DMPO eliminated the Fe-PCH inducted artifacts. When neutrophils were stimulated with PMA in the presence of DTPA, DMSO, Fe-PCH and PBN, a nitroxide species consistent with PBN/$\cdot OCH_3$ was detected, indicating $\cdot OH$ formation (Fig. 5 *A*). The omission of PMA (Fig. 5 *B*), or the inclusion of catalase (Fig. 5 *C*), prevented PBN/$\cdot OCH_3$ formation. The substitution of Fe-PVD for Fe-PCH failed to promote PBN/$\cdot OCH_3$ formation (Fig. 5 *D*).

PMA stimulation may lead to preferential secretion of secondary granule contents (56). To optimize exposure of Fe-PVD to primary granule proteases experiments were repeated in which neutrophils which had been pretreated with dihydrocytochalasin B to prevent phagosome closure (57) were stimulated with opsonized zymosan in the presence of Fe-PVD, DMSO, DTPA, and DMPO or PBN. Once again no spin-trap evidence of $\cdot OH$ formation was detected (not shown).

Similar to the approach using the hypoxanthine/xanthine oxidase system the above experiments were repeated using formation of TBA-reactive deoxyribose oxidation products as an indicator of $\cdot OH$ generation. Chromogen formation was seen when neutrophils were stimulated with PMA in the presence of Fe-PCH but not Fe-PVD (Table II).

Both PCH and PVD are unable to bind Fe^{2+} (33, 34, 58).

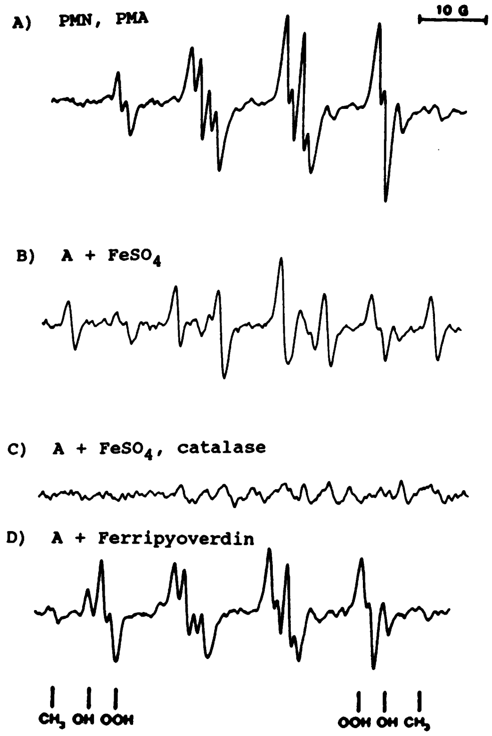

Figure 4. Representative ESR spectra (n = 3–5) of solutions containing: (A) neutrophils (5 × 10^6/ml), DMSO (140 mM), DMPO (100 mM), DTPA (0.1 mM), and PMA (0.1 μg/ml); (B) contents of A plus $FeSO_4$ (0.1 mM); (C) contents of A plus $FeSO_4$ (0.1 mM) and catalase (500 U/ml); and (D) contents of A plus Fe-PVD (0.1 mM). Location of high- and low-field peaks corresponding to DMPO/$\cdot CH_3$, DMPO/$\cdot$OH, and DMPO/$\cdot$OOH are indicated as *CH_3, OH,* and *OOH,* respectively. ESR spectrometer settings were as in Fig. 1.

Therefore, $\cdot O_2^-$ mediated reduction of Fe^{3+} bound to either PCH or PVD should result in release of free Fe^{2+} that would then be available for oxidation by H_2O_2. Documentation of such an event would suggest a possible means for interrupting redox cycling of iron bound to the siderophore, either by irreversibly binding the free Fe^{2+} or through the addition of competing Fe^{3+} chelators to limit reassociation of Fe^{3+} with the siderophore.

Ferrozine avidly binds Fe^{2+} forming a complex with a peak absorbance at 562 nm (40). The formation of this complex in a solution containing ferrozine, Fe-PCH or Fe-PVD, and a source of $\cdot O_2^-$ would reflect the ability of $\cdot O_2^-$ to reduce, and cause the release of, siderophore bound Fe^{3+}. Addition of xanthine oxidase to a solution of hypoxanthine, ferrozine, and Fe-PCH resulted in a gradual increase in A_{562} (Fig. 6). No increase in A_{562} was seen with Fe-PVD, apopyochelin, or the omission of xanthine oxidase. These data confirm that $\cdot O_2^-$ can reduce and release iron bound to Fe-PCH but not Fe-PVD.

Discussion

Infection with *Pseudomonas aeruginosa* is associated with local accumulation of leukocytes and leads to both acute and chronic damage to surrounding tissues (37, 59, 60). In a variety of inflammatory conditions, local tissue injury may be related to generation of the highly reactive oxidant $\cdot$OH (1, 4–7), formed through the reaction of neutrophil-derived $\cdot O_2^-$ and H_2O_2 with an iron catalyst (2). Because of its possible implications for *Pseudomonas*-associated tissue damage we investigated whether the *Pseudomonas* siderophores PCH and PVD bind iron in a manner that promotes $\cdot$OH formation when $\cdot O_2^-$ and H_2O_2 are present.

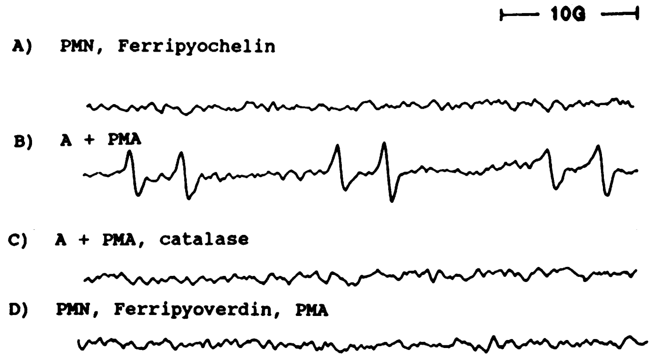

Figure 5. Representative ESR spectra (n = 5) of solutions containing: (A) neutrophils (5 × 10^6/ml), DMSO (140 mM), PBN (10 mM), DTPA (0.1 mM), and Fe-PCH (0.2 mM); (B) contents of A plus PMA (0.1 μg/ml); (C) contents of A plus PMA (0.1 μg/ml) and catalase (500 U/ml); (D) contents of B except Fe-PVD (0.1 mM) was substituted for Fe-PCH. The species observed in B is that of PBN/$\cdot OCH_3$. ESR spectrometer settings were as in Fig. 2.

Three separate approaches were used to measure $\cdot$OH production, two spin-trapping systems and the deoxyribose oxidation assay. Regardless of methodology, when Fe-PCH was added to an enzymatic $\cdot O_2^-$- and H_2O_2-generating system evidence of $\cdot$OH production was detected. Iron complexed to PVD by contrast did not appear to promote $\cdot$OH formation. Inhibition of Fe-PCH catalyzed $\cdot$OH generation by inclusion of catalase or SOD in the system is consistent with formation of this species by a Haber–Weiss mechanism. Although previously hypothesized (61), to our knowledge this is the first report of the ability of iron bound to a bacterial siderophore to be capable of catalyzing the Haber–Weiss reaction.

Central to the hypothesis that siderophore bound iron can participate in Haber–Weiss catalysis is that the ferric iron can be reduced to ferrous iron by $\cdot O_2^-$ with subsequent reoxidation to the ferric state by H_2O_2. Consistent with previous work suggesting that PCH could not bind Fe^{2+} (33, 34, 58, 62) inclusion of ferrozine and Fe-PCH in an enzymatic $\cdot O_2^-$-gener-

Table II. Ability of Pyochelin and Pyoverdin to Catalyze Hydroxyl Radical Formation by Stimulated Neutrophils (PMN)

	A_{532}
PMN + Fe-EDTA	0.532
PMN + Fe-EDTA + catalase	0.018
PMN + Fe-EDTA + SOD	0.008
PMN + ferripyochelin	0.120
PMN + ferripyochelin + catalase	0.001
PMN + ferripyochelin + SOD	0.019
PMN + ferripyoverdin	0.003

Formation of TBA-reactive deoxyribose oxidation products measured as A_{532} representative of seven separate experiments resulting from the stimulation of human neutrophils (PMN) by PMA in the presence of the iron chelates noted.

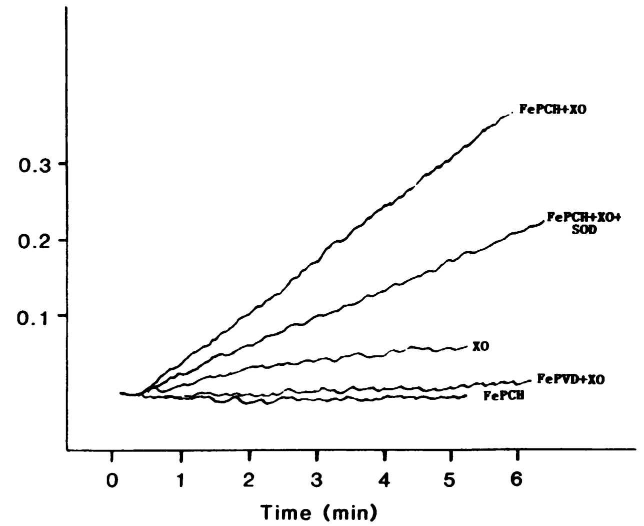

Figure 6. Increase in A_{562} over time reflecting formation of Fe^{2+}-ferrozine complex after addition of xanthine oxidase to a mixture of hypoxanthine and ferrozine alone (labeled XO) and in the presence of Fe-PCH (*Fe-PCH + XO*) or Fe-PVD (*Fe-PVD + XO*). Also shown are results with the addition of SOD to the Fe-PCH/xanthine oxidase mixture (*Fe-PCH + XO + SOD*) and with a mixture of hypoxanthine, ferrozine, and Fe-PCH to which xanthine oxidase was not added (Fe-PCH). Results are representative of three to four experiments.

ating system resulted in formation of the Fe^{2+}-ferrozine complex due to $\cdot O_2^-$-mediated release of PCH-bound iron. Thus Fe–PCH-induced $\cdot OH$ generation likely involves the interaction of free Fe^{2+} with H_2O_2 to yield $\cdot OH$ and Fe^{3+} which then can either reassociate with the siderophore or be reduced again by $\cdot O_2^-$. Alternatively, we found that the reaction of H_2O_2 directly with Fe-PCH may also yield a species resembling $\cdot OH$. Similar results have been reported with other ferric iron chelates (49–51) but the mechanism of this reaction remains in doubt.

It has been suggested that reaction of H_2O_2 with Fe^{2+} under some circumstances may yield an oxidant species which is not $\cdot OH$ but rather an Fe^{2+}-H_2O_2 complex (ferryl species) (52, 63, 64). We are unable to eliminate the possibility that the species catalyzed by Fe-PCH is an $\cdot OH$-like species rather than $\cdot OH$ itself. However since each of these oxidants is highly reactive, from a biologic standpoint it may not be a critical point.

In vivo the most important source of $\cdot O_2^-$ and H_2O_2 would be stimulated phagocytes, particularly neutrophils. In addition to $\cdot O_2^-$ and H_2O_2 release, a wide array of enzymes and proteins are also released during neutrophil stimulation (53). Previous reports have found that at least two of the granular components affect the potential for $\cdot OH$ formation in association with the neutrophil respiratory burst. Lactoferrin and myeloperoxidase inhibit $\cdot OH$ formation by iron-supplemented neutrophils by sequestering iron in a noncatalytic form and consuming H_2O_2, respectively (15, 20, 54). In contrast, it seemed possible that neutrophil proteases through their action on the peptide PVD could alter the potential of iron bound to PVD to participate in the Haber–Weiss reaction.

However, in spite of these theoretical considerations using the same assays employed with the hypoxanthine/xanthine oxidase system, we again found that only Fe-PCH would catalyze $\cdot OH$ production by stimulated PMN. By inference, neutrophil proteases do not endow Fe-PVD with catalytic properties and lactoferrin and myeloperoxidase release do not eliminate Fe–PCH-catalyzed $\cdot OH$ generation. An alternative explanation for the apparent lack of $\cdot OH$ formation with the coincubation of Fe-PVD and stimulated neutrophils would be if Fe-PVD inhibited the neutrophil respiratory burst. However, as assessed by either oxygen consumption or ferricytochrome *c* reduction no such inhibition was detected (results not shown).

Although our data clearly suggest that Fe-PCH is capable of catalyzing formation of $\cdot OH$ in the presence of neutrophil or enzymatic sources of $\cdot O_2^-$ and H_2O_2 it remains unclear as to the relevance of such an observation to in vivo conditions. Indirect evidence has been presented that *Pseudomonas* secrete PVD and PCH in vivo (38). However, no data are available as to what the concentration of either siderophore may be at sites of *Pseudomonas* infection. We are currently developing assay systems to quantitate levels of PCH and PVD in biologic fluids (e.g. bronchoalveolar lavage samples). Nevertheless, the concentration of siderophores used in this study were the same or less than those which accumulated routinely, in in vitro broth culture of *P. aeruginosa* (33, 34), providing some evidence of biologic relevance.

Assuming that concentrations of Fe-PCH sufficient to generate biologically relevant amounts of $\cdot OH$ are present at sites commonly involved in pseudomonas infection (e.g., lung) the potential of such $\cdot OH$ to damage local tissue is unclear. $\cdot OH$ is an extremely reactive oxidant. If formed in vivo it would likely diffuse only a few angstroms before encountering an oxidizable biomolecule. Consequently to be involved in injury to host cells, formation of $\cdot OH$ by the above mechanism would likely need to occur in close proximity to host cell membrane. PCH is very lipophilic (33), and we have obtained preliminary evidence that Fe-PCH readily becomes associated with eukaryotic cell membranes (Coffman, T. J., and B. E. Britigan, unpublished). Such targeting of catalytic iron to host membrane could markedly enhance Fe-PCH toxicity. We are currently examining whether the presence of Fe-PCH enhances the susceptibility of relevant eukaryotic cells (e.g., pulmonary epithelial cells) to $\cdot O_2^-/H_2O_2$-mediated cytotoxicity.

A variety of extracellular secretory products of *P. aeruginosa* have been incriminated in the tissue destruction observed in *Pseudomonas* infection (37, 59, 60). Our finding that the pseudomonas siderophore Fe-PCH catalyzes $\cdot OH$ formation suggests another possible and novel mechanism for inflammatory damage may be present. Some 50 other siderophores produced by an assortment of bacteria and fungi have been identified (29). Ability to form siderophores has been suggested as a virulence factor for some bacterial pathogens other than *P. aeruginosa* (65, 66). Evaluation of other microbial siderophores may result in the identification of as yet other such compounds capable of serving as Haber–Weiss catalysts.

It has previously been reported that *Staphylococcus aureus* grown in vitro so as to enhance its intracellular iron stores is more susceptible to killing by H_2O_2 and human monocytes, but not neutrophils (67–69). These data have been interpreted as evidence for the involvement of $\cdot OH$ catalyzed by bacteria-associated iron in phagocyte microbicidal activity and inflammatory tissue injury. In recent work we have been unable to document formation of $\cdot OH$ using spin trapping techniques following incubation of similarly prepared iron-rich *Staphylococci* with the hypoxanthine/xanthine oxidase system, monocytes, or neutrophils (Cohen, S. M., B. E. Britigan, J. S. Chai, T. L. Roeder, and G. M. Rosen, manuscript submitted for publication). The work reported in the present communication may also have somewhat greater relevance to in vivo conditions than that with iron-rich organisms. In general, sites of bacterial infection are felt to be low-iron microenvironments from the standpoint of the microorganism (21, 31, 32, 38). Thus, in vivo, iron-rich organisms would be unlikely to occur while this environment would induce bacterial production and secretion of siderophores such as PCH (29, 30, 33, 34, 36). In addition, with regard to inflammatory tissue injury, catalytic iron associated with bacteria would be expected to result in generation of ·OH in the immediate proximity of the organism. Given the limited diffusibility of ·OH this would make it less likely to damage surrounding tissue. Siderophores on the other hand are freely diffusible, allowing them potentiality to target catalytic iron to host cells.

In summary, we have obtained experimental evidence that iron bound to one of two pseudomonas siderophores, PCH, is capable of in vitro catalysis of the Haber–Weiss reaction. Such a process in vivo could contribute to tissue injury associated with *P. aeruginosa* infection. Further work supportive of such a hypothesis could suggest new means to limit tissue injury associated with *P. aeruginosa* and possibly other bacterial infections.

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