Oxidation of *Escherichia coli* Iron Centers by the Myeloperoxidase-mediated Microbicidal System*

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Myeloperoxidase, H$_2$O$_2$, and a halide (chloride, bromide, iodide) constitute a powerful microbicidal system which is active against a wide variety of microorganisms and is believed to contribute to the antimicrobial activity of neutrophils. The precise mechanism by which this system exerts its toxicity is unknown. We report here that the microbicidal activity of the myeloperoxidase-H$_2$O$_2$-chloride system on *Escherichia coli* is associated with the loss of iron into the medium as measured by the release of $^{69}$Fe from prelabeled organisms. Iron loss (but not bactericidal activity) was considerably increased by the addition of EDTA or other iron chelators; it was not associated with a corresponding release of protein or labeled compounds from organisms prelabeled with $^{14}$C-amino-acids. Iron loss was observed with chloride or bromide as the halide, but not when iodide was employed in microbicidal concentrations. Microbicidal activity was detected at an earlier time period and at a lower halide concentration than was iron loss. Analogous changes were observed when cytchrome c was oxidized by the myeloperoxidase H$_2$O$_2$-halide system. The initial response was a shift in the Soret maximum, followed by a fall in absorbance accompanied by the loss of iron. As with the intact organism, iron loss was evident with chloride and bromide, but not with iodide as the halide. These findings suggest that microbial iron centers are a target for the myeloperoxidase-mediated antimicrobial system and that their oxidation may contribute to microbicidal activity.

Polymorphonuclear leukocytes efficiently phagocytose and kill a variety of microorganisms (for review, see Refs. 1–6). Among the many microbicidal systems which contribute to this process is one which depends upon the granule enzyme myeloperoxidase, H$_2$O$_2$ (generated by the leukocyte or, in some instances, by the ingested organism), and a halide (7–9). In general, myeloperoxidase reacts with H$_2$O$_2$ to form an enzyme-substrate complex (or complexes) which oxidizes the halide to a powerful microbicidal product. The nature of the product formed varies with the halide employed. Hypochlorous acid is believed to be the primary oxidant formed by the chloride-dependent system (7–9); hypobromous acid is presumed to be formed from bromide, whereas the oxidants formed by the iodide-dependent system are less certain. These primary products of halide oxidation may react with cellular constituents to generate additional toxic derivatives such as aldehydes and chloramines (8, 9).

It is presumed that the powerful oxidants formed by the myeloperoxidase system can attack the microorganism at various sites and in a variety of ways; the precise nature of this attack however is unknown. Recently, hypochlorous acid, the primary product of the myeloperoxidase-H$_2$O$_2$-chloride system, was found to oxidize a number of compounds which were structurally related to biologically important components of bacterial cells (10). Among the most sensitive compounds are those that contain iron centers. This prompted a study of the ability of the myeloperoxidase system to oxidize critical iron centers in the microorganism. The myeloperoxidase system was found to dramatically alter the iron content of *Escherichia coli* and studies with cytchrome c as a model suggested that heme proteins are one of the groups of compounds with iron centers which are susceptible to attack.

**MATERIALS AND METHODS**

*Special Reagents—Myeloperoxidase was prepared from canine pyometral pus by the method of Agner (11) to the end of step 6 and assayed by the $0$-dianisidine method (12). One unit of enzyme is the amount decomposing 1 mmol of H$_2$O$_2$/min at 25 °C. Bathophenanthroline sulfonate, nitritotriacetic acid, diethylenetriaminepentaacetic acid, EDTA, cytchrome c (Type VI, from horse heart), and glucose oxidase (Type V, from Aspergillus niger, 1490 units/ml) were obtained from Sigma and desferoxamine mesylate from Ciba Pharmaceutical Co., Summit, NJ.

*Microorganisms—Escherichia coli* (ATCC No. 11775; American Type Culture Collection, Rockville, MD) was maintained on blood agar plates and grown overnight in liquid growth media at 37 °C prior to use. The growth media employed were Trypticase soy broth (Baltimore Biological Laboratories, Baltimore, MD) or medium A containing 7 g of KH$_2$PO$_4$, 3 g of KH$_2$PO$_4$, 0.5 g of sodium citrate, 2H$_2$O, 0.1 g of MgSO$_4.7$H$_2$O, 1 g of (NH$_4$)$_2$SO$_4$, and 1 g of sodium succinate/liter (13). The iron concentration of Trypticase soy broth was 11 μg (atomic absorption kindly performed by Dr. Michael Albrich, Oregon Graduate Center, Beaverton, OR) and that of medium A was 1.8 μg by colorimetric determination (14). With Trypticase soy broth, growth was in loosely capped screw top 20-ml glass test tubes approximately three-quarters full. With medium A, growth was in loosely capped polystyrene tissue culture flasks with a large surface area (75cm$^2$) relative to the depth of the medium (2 mm) to facilitate aeration. Unless otherwise indicated, organisms were grown in Trypticase soy broth. Following growth, the organisms were washed twice by centrifugation and suspended in 0.1 M sodium sulfate to the required optical density at 540 nm. Stock suspensions were diluted 10-fold in the final reaction mixture.

Organisms were isotopically labeled by overnight culture in either Trypticase soy broth or medium A supplemented with $^{35}$FeCl$_3$ (Amer- sham) or U-$^{14}$C-amino-acids (NEC 445, New England Nuclear). Activity in the growth media ranged from 3 to 15 μCi/ml.

**Microbicidal Activity**—The components indicated in the legends to the figures and tables were incubated in plastic tubes (12 × 75 mm) in a 37 °C water bath shaker oscillating 80 times/min. At the times indicated, 0.1-ml aliquots were removed, serial dilutions were prepared in 0.1 M sodium sulfate, and the viable cell count was deter-
mixed by the pour plate method using Trypticase soy agar. The absence of colonies on plating 0.1 ml of the undiluted incubation mixture is designated zero organisms per ml (actually < 10). Loss of Radioactive Components from E. coli—Following incubation as described in the legends to the figures and tables, the reaction mixture was passed through a membrane filter with a 0.45-µm diameter (Type HA, Millipore Corp., Bedford, MA) which retained the microorganisms. Activity in a defined volume of filtrate, usually 0.5 ml, was compared to that in an equal volume of unfiltered suspension. Preliminary experiments with 56Fe-labeled organisms indicated that the combined activity of filtrate and filtrate accounted for 102 ± 2% (mean ± S.D., N = 24) of total added activity.

Lipopolysaccharide Release from E. coli—E. coli (5 × 10^7/ml) were incubated in 10^{-5} M sodium sulfate, 0.1 M sodium chloride, and 4 × 10^{-5} M sodium acetate buffer, pH 5.0, for 1 h at 37 °C. EDTA (10^{-2} M) was added where indicated. One-ml samples were centrifuged at room temperature for 4 min at 8700 × g (Microfuge B, Beckman Instruments Inc., Palo Alto, CA) and the supernatant was filtered through a 0.45-µm pore size membrane filter. The lipopolysaccharide content of the supernatant was determined by its content of thiobarbituric acid-reactive sugars (15, 16). Briefly, 0.63 ml was acidified with 0.07 ml of 2 N H2SO4 and hydrolyzed in capped tubes for 15 min at 95-100 °C water bath. The hydrolysate was oxidized with 0.1 ml of 0.025 N periodic acid in 0.125 N H2SO4 for 20 min at 56 °C and the reaction was stopped with 0.2 ml of 25% sodium arsenite in 0.5 N HCl. Two ml of 0.6% thiobarbituric acid in 0.007 N NaOH was added and the mixture was allowed to react for 20 min at 95-100 °C. The samples were clarified by centrifugation at room temperature and the absorbance at 532 nm determined. Suspension medium without organisms, processed in the same manner, served as the blank. The lipopolysaccharide content of the mixture supernatant was compared to that of the whole cell suspension and the results were expressed as the percent released.

Spectral Changes and Loss of Iron from Cytochrome c—The reaction mixture as described in the legend to Fig. 3 was incubated at 37 °C and, at the indicated intervals, 1.0-ml aliquots were transferred to spectrophotometer cuvettes (1 cm path length) and the spectrum was recorded between 600 and 350 nm using a Cary Model 14 spectrophotometer (Cary Instruments, Monrovia, CA). The release of iron was determined using 1.0-ml aliquots from separate tubes containing the same components. The aliquots were added to spectrophotometer cuvettes and the components were reduced with a few crystals of sodium dithionite. Absorbance was determined at 535 nm before and after the addition of 0.01 ml of 0.01 M bathophenanthroline sulfonate and the absorbance change was compared to a standard curve prepared by adding ferrous sulfate (0-10 µM) to a reaction mixture containing 10 µM cytochrome c, 0.1 M NaCl, and 0.04 M sodium acetate, pH 5.0.

Protein Content—Protein was determined by the method of Lowry et al. (17) using human serum albumin as the standard. Statistical Analysis—Analysis of the microbial viability data was as previously described (18). Other data were analyzed by Student's two-tailed t-test (not significant, p > 0.05).

RESULTS

Intact Organisms—Incubation of 5 × 10^7 E. coli with the complete myeloperoxidase-H2O2-chloride system for 1 h under the conditions employed in Table I produced a marked fall in viable cell count, which was associated with an increase in microbial iron release into the medium from 3 to 12% (p < 0.01). Supplementation of the antimicrobial system with EDTA dramatically increased iron loss to 70% (p < 0.001) without altering significantly either microbicidal activity or spontaneous iron loss. Deletion of myeloperoxidase, H2O2, and chloride from the EDTA-supplemented system abolished microbial activity and reduced iron loss to control levels. The 56Fe lost from E. coli was not precipitated by either 5% trichloroacetic acid or 50% methanol. H2O2 could be replaced by the H2O2-generating system glucose + glucose oxidase with comparable results (data not shown).

We considered two possible mechanisms for the EDTA stimulation of iron release. First, EDTA can disrupt the integrity of the E. coli outer membrane with loss of lipopolysaccharide into the suspension medium. Under these conditions,

| Supplements | Viable cell count organisms/ml | % 56Fe release |
|-------------|-------------------------------|---------------|
| Control     | 5.1 ± 3 ± 0°                   |               |
| Control + EDTA (7) | 3.9 ± 5 ± 1 |               |
| Complete system (2) | 0.0° ± 12° 1' |               |
| Complete system + EDTA (7) | 0.000004° ± 70 ± 1 |               |
| Myeloperoxidase omitted (3) | 5.1° ± 5° 0' |               |
| H2O2 omitted (3) | 5.0° ± 4° 0' |               |
| Cl− omitted (3) | 4.2° ± 4° 0' |               |

a Geometric mean.

b Arithmetic mean ± S.E.

* Significantly different from Control (or Control + EDTA), p < 0.02.

* Significantly different from Complete system + EDTA, p < 0.001.

TABLE I
Iron release associated with the bactericidal activity of the myeloperoxidase system

The complete system contained 0.04 M sodium acetate buffer, pH 5.0, 5 × 10^{-3} M Fe-labeled E. coli, 0.1 M sodium chloride, 16 milligrams of myeloperoxidase, and 5 × 10^{-3} M H2O2 in a final volume of 1.0 ml. Myeloperoxidase and H2O2 were omitted from control tubes and 10^{-4} M EDTA was added where indicated. Incubation was for 90 min at 37 °C. The number of experiments is shown in parentheses.

TABLE II
Effect of chelators on myeloperoxidase-mediated iron loss from E. coli

Incubation conditions were as described for the complete system in Table I except that chelators and magnesium sulfate were added where indicated. Results are the mean ± S.E. of 3 experiments.

% 56Fe release

| Chelator | Complete myeloperoxidase system | Buffer alone |
|---------|-------------------------------|-------------|
| None    | 7 ± 1 ± 3 ± 0°                 | 7 ± 1       |
| EDTA, 10^{-4} M | 71 ± 3 ± 5 ± 1 | 71 ± 3      |
| EDTA, 10^{-4} M + MgSO4, 10^{-3} M | 67 ± 0      | 67 ± 0      |
| Diethylenetriaminepentaacetic acid, 10^{-4} M | 68 ± 4 ± 3 ± 1 | 68 ± 4      |
| Desferrioxamine, 10^{-4} M | 66 ± 5 ± 7 ± 1 | 66 ± 5      |
| Citrate, 10^{-4} M | 65 ± 4 ± 7 ± 2 | 65 ± 4      |
| Nitribriacetate, 10^{-4} M | 74 ± 3 ± 5 ± 0 | 74 ± 3      |

The outer membrane becomes abnormally permeable to macromolecules (19). This action may render internal membrane proteins more susceptible to attack by the myeloperoxidase system with increased iron release. Alternatively, EDTA may function as an iron chelator, thus maintaining the released iron in solution.

Two findings militate against the first mechanism. Lipopolysaccharide release from E. coli during a 1-h incubation at pH 5.0 under the conditions described under "Materials and Methods" (10 ± 2%; n = 4) was not significantly increased by the addition of 10^{-4} M EDTA (15 ± 3%; n = 4) suggesting that outer membrane disruption is not a prominent effect of EDTA under these conditions. Furthermore, Mg2+, which inhibits the outer membrane effects of EDTA (19), did not decrease the EDTA effect on myeloperoxidase-mediated microbial iron loss, even at a Mg2+/EDTA ratio of 16:1 (Table II). A number of iron chelators (diethylenetriaminepentaacetic acid, desferrioxamine, citrate, nitribriacetate) were examined for their ability to replace EDTA in the stimulation of iron release by the myeloperoxidase-mediated system. All were effective in this regard, whereas none appreciably increased iron release from E. coli incubated in buffer alone (Table II). None of the chelators studied were microbicidal alone nor did they interfere with the microbicidal activity of the complete myeloperoxidase-H2O2-chloride system (data not shown).
These findings suggest that the property of EDTA which is important in mediating optimal microbial iron loss is its ability to chelate iron.

Fig. 1 compares the kinetics of iron loss induced by the myeloperoxidase-H$_2$O$_2$-chloride-EDTA system to that of microbicidal activity. The fall in viable cell count was essentially complete in 5 min whereas iron loss, although significantly increased at 5 min ($p < 0.01$), was only 5% above background. Iron loss increased rapidly with further incubation, although it lagged behind in viable cell count.

Chloride can be replaced by bromide or iodide as the halide requirement for the myeloperoxidase-mediated antimicrobial system (9). Fig. 2 compares microbicidal activity and iron loss for each halide over a range of concentrations. Bactericidal activity was lost when no halide was added, whereas the iron loss induced by myeloperoxidase + H$_2$O$_2$ ($17 \pm 4\%$) remained significantly greater than the buffer control ($6 \pm 1\%$, $p < 0.001$). When chloride was added (Fig. 2A), a bactericidal effect was detected at a concentration of 10$^{-7}$ M ($p < 0.01$) which increased to a maximum at 10$^{-5}$ M. Iron loss was not increased by chloride at 10$^{-4}$ M but, as with bactericidal activity, rose sharply as the chloride concentration was increased above that level. Bromide (Fig. 2B) was considerably more effective than chloride, with a major fall in viable cell count and increase in iron release occurring when the bromide concentration was raised from 10$^{-6}$ to 10$^{-3}$ M. At 10$^{-6}$ M, bactericidal activity was significantly increased ($p < 0.01$), whereas iron loss was not. Iodide (Fig. 2C) was more effective than bromide or chloride as the halide required for bactericidal activity, with the fall in viable cell count increasing sharply as the iodide concentration was raised above 10$^{-7}$ M. However, in sharp contrast to bromide or chloride, increased iron release was not observed when iodide was employed at concentrations (10$^{-6}$-10$^{-4}$ M) which were markedly bactericidal.

The loss of iron on exposure of E. coli to the myeloperoxidase system was not a manifestation of the general loss of cellular constituents. The total protein content of the E. coli suspensions employed in these studies (5 x 10$^8$ organisms/ml) was 23 mg/ml as measured by the Lowry technique. Protein loss from E. coli incubated with the myeloperoxidase + H$_2$O$_2$ + Cl$^-$ + EDTA system under the conditions employed in Table I lost 4.0 + 0.7% (n = 4) of the label as compared to 2.1 ± 0.5% (n = 4) for organisms incubated in the absence of myeloperoxidase and H$_2$O$_2$. The radioactivity released was not precipitable with cold 5% trichloroacetic acid suggesting that the labeled material was not protein. These findings suggest that little or no protein or other labeled metabolite was released by the myeloperoxidase system under conditions in which there was extensive iron loss.

**Cytochrome c**—Fig. 3 demonstrates the spectral changes induced by incubation of cytochrome c with myeloperoxidase, glucose, glucose oxidase (as a continuous source of H$_2$O$_2$), and chloride. The first change noted was a shift in the Soret maximum from 410 to 400 nm, with little loss of intensity. This shift in the Soret band, which was also accompanied by a loss of absorbance at 530 nm, occurred within the first 5 min of incubation under our experimental conditions. With continued incubation, there was a progressive loss of absorbance in the Soret region accompanied by a slight shift in the maximum to 405 nm. Bleaching of the cytochrome c was almost complete at 60 min.

The spectral changes induced by the peroxidase system were associated with the release of iron in a form which was accessible to chelation by bathophenanthroline sulfonate. The
glucose oxidase, 80 milliunits of myeloperoxidase, 0.1 ride, and "Materials and Methods." The release of iron indicated intervals, 1.0-ml aliquots were removed and absorbance was 0.04 at 400 and the peroxidase system. The reaction mixture was with the halide system, as measured by the fall in absorbance at 410 nm, was observed with chloride (10^{-2}–10^{-1} M) and bromide (10^{-4}–10^{-1} M) as the halide (Fig. 4); however, as with iron loss from E. coli, iodide was ineffective at microbicidal concentrations (10^{-8}–10^{-7} M).

**DISCUSSION**

Incubation of suspensions of E. coli with myeloperoxidase, H_{2}O_{2}, and a halide results in both a fall in viability as measured by colony-forming units and by the disruption of iron structure as measured by the leakage by labeled microbial iron into the suspension medium.

The mechanism of iron loss is incompletely understood. It does not appear to result from a major disruption of the surface structure since iron loss is not associated with a corresponding release of proteins or labeled compounds from organisms prelabeled with ^14C-amino-acids. This suggests the penetration of the myeloperoxidase system or its products to iron protein sites and the relatively selective oxidation of these proteins with the release of iron. A likely source, at least in part, of the iron released into the medium is the cytoplasmic membrane electron transport chain which contains a substantial concentration of iron proteins (20). Potential physical and chemical barriers to the penetration of the myeloperoxidase system or its derived oxidants exist in the periplasmic space, the cell wall, and the outer membrane which are peripheral to the cytoplasmic membrane. While the permeability characteristics of the E. coli outer membrane would be expected to preclude the penetration of a protein such as myeloperoxidase (21), hypochlorous acid or other halide-derived oxidants appear to be able to diffuse to the cytoplasmic membrane and react with its components. Thus, reagent hypochlorous acid irreversibly oxidizes heme-containing cytochromes in intact E. coli and stops microbial respiration (10) and the permeability characteristics of the cytoplasmic membrane are altered by the peroxidase-H_{2}O_{2}-halide system (22).

Microbial iron loss did not correlate precisely with microbicidal activity. Thus, iron loss lagged behind the loss of viability and when the bromide and chloride concentrations were decreased, a level was reached at which iron loss was undetectable while microbicidal activity, although greatly reduced, was still evident. Iron loss into the medium was increased by the addition of a chelating agent (EDTA, diethylenetriaminepentaacetic acid, desferrioxamine, citrate, or nitrotoluidate), whereas microbicidal activity was not. Finally, when iodide was employed as the halide, there was no detectable iron loss under conditions in which a major microbicidal effect was observed.

This lack of precise correlation between iron loss and loss of viability may be due to a number of factors. First, damage to microbial iron may be a late event unrelated to the primary chemical damage leading to cell death. Alternatively, damage to metabolically active iron centers may occur early but release of iron may be a relatively late consequence of that damage. When cytochrome c was exposed to the peroxidase system, the initial response was a shift in the Soret maximum and the loss of the 530 nm peak, and only with further incubation was there a fall in absorbance in the Soret region and the appearance of free iron in the medium. Thus, with this model compound, changes in the heme center were evident prior to the loss of iron. An additional possibility is the early oxidation of iron centers followed by a delay in the passage of the released iron into the medium. The complex microenvironment of iron proteins embedded in the lipid inner membrane might be expected to limit the movement of iron. Further, if oxidized iron is initially released into the interior of the cell, the neutral or slightly alkaline pH that exists there (23) would render ferric iron highly insoluble and thus contribute to a delay in the passage of iron into the external medium. The need for a chelating agent for optimum iron loss

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**Fig. 3. Bleaching of cytochrome c by the myeloperoxidase system with the loss of heme iron.** The reaction mixture contained 0.04 M sodium acetate buffer, pH 5.0, 0.01 M glucose, 0.3 unit of glucose oxidase, 80 milliunits of myeloperoxidase, 0.1 M sodium chloride, and 10^{-5} M ferricytochrome c in a total volume of 5.0 ml. At the indicated intervals, 1.0-ml aliquots were removed and absorbance was determined between 550 nm and 370 nm. The change in absorbance at 400 and 410 nm as a function of time (inset) was taken from these scans. The release of iron (inset) was determined as described under "Materials and Methods."

**Fig. 4. Effect of halides on the bleaching of cytochrome c by the peroxidase system.** The reaction mixture was as described in Fig. 3 except that the final volume was 1.0 ml, myeloperoxidase and glucose oxidase were reduced proportionately, and 0.1 M sodium chloride was replaced by chloride, bromide, or iodide (sodium salts) at the concentrations indicated. Sodium sulfate was added to maintain osmolality when the halide concentration was below 0.1 M. Absorbance at 410 nm was determined after 10 min of incubation at 37 °C. Results are the means of 1-5 determinations. *, significantly different from control, p < 0.05.

Inset in Fig. 3 compares the rate of heme iron release to that of the bleaching of cytochrome c as measured by the change in absorbance at 410 and 400 nm. Iron release began after a 5-min lag period and reached a value of 45% of the total heme iron by 60 min. The fall in absorbance at 410 nm began immediately and was 77% complete at 60 min. In contrast, the absorbance at 400 nm initially rose due to the shift in the Soret band, began to fall at about 5 min, and was 76% complete at 60 min. Bleaching of cytochrome c by the peroxidase system, as measured by the fall in absorbance at 410 nm, was observed with chloride (10^{-2}–10^{-1} M) and bromide (10^{-4}–10^{-1} M) as the halide (Fig. 4); however, as with iron loss from E. coli, iodide was ineffective at microbicidal concentrations (10^{-8}–10^{-7} M).
supports a requirement for the solubilization of the released iron. Finally, cell death may result from the oxidation of a relatively small number of iron centers and the amount of iron released under these conditions may be below the levels reliably detected by our assay.

Our evidence would suggest that with iodide as the halide, iron loss as measured by its release into the medium is not a crucial requirement for microbicidal activity. Although it is possible that the iodide system, perhaps through iodination, forms a barrier to the diffusion or iron into the medium, it seems more likely that there is a fundamental difference in the effects of the peroxidase system on the microbe, depending on the halide. It is of interest in this regard that, under our conditions, the iodide system fails to bleach cytochrome c in contrast to the bromide- or chloride-dependent systems.

It appears likely that damage to microbial iron structures contributes to myeloperoxidase-mediated microbicidal activity. Heme and non-heme iron proteins are essential to the normal function of the microbial electron transport chain and extensive damage to these structures would be incompatible with survival of the cells. Oxidation of other essential components of the microbe (e.g. sulfhydryl enzymes (24)) also would be expected to contribute to the death of the organism, with the crucial hit (or combination of hits) varying with the target organism and the conditions of attack. While release of iron is certainly evidence of damage to the cell and is possibly a consequence of the lethal hit in some circumstances, it may have an additional function in the phagosome, that of providing iron for myeloperoxidase-independent antimicrobial systems (25, 26). It is now the prevailing view that among the antimicrobial systems of the phagocyte is one which depends on hydroxyl radicals (OH-) and two mechanisms have been proposed for their generation in the phagosome, both requiring iron. In one, Fe^{3+} reacts stoichiometrically with H₂O₂ to form OH- (Fenton's reagent) and, in the second, iron acts catalytically, being oxidized by H₂O₂ and reduced by superoxide, with the overall reaction leading to the generation of OH- (Haber-Weiss reaction) (for review, see Refs. 2, 3, 5, and 6). One of the sources of this iron may be microorganisms oxidized by the myeloperoxidase-H₂O₂-chloride (or bromide) system.

Acknowledgments—We thank Drs. J. Michael Albrich, Daniel R. Ambruso, Peter Elsbach, James K. Hurst, Richard B. Johnston, Jr., and Jerrold Weiss for helpful discussion, Joanne Fluvog and Douglas J. Evans for expert technical assistance, and Linda Knudsen for help in the preparation of the manuscript.

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