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The inhibition of bacterial growth by hypochlorous acid

Possible role in the bactericidal activity of phagocytes

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The 'respiratory burst' of phagocytes such as neutrophils generates superoxide which forms H_2O_2 by dismutation. H_2O_2 and Cl^- ions serve as substrates for the enzyme myeloperoxidase to generate hypochlorous acid (HOCl). HOCl is thought to play an important role in bacterial killing, but its mechanism of action is not well characterized. Furthermore, although many studies *in vitro* have shown HOCl to be a damaging oxidant with little or no specificity (particularly at high concentrations), bacteria which have been ingested by phagocytes appear to experience a rapid and selective inhibition of cell division. Bacterial membrane disruption, protein degradation, and inhibition of protein synthesis, do not seem to occur in the early phases of phagocyte action. We have now found that low concentrations of HOCl exert a rapid and selective inhibition of bacterial growth and cell division, which can be blocked by taurine or amino acids. Only 20 μM-HOCl was required for 50% inhibition of bacterial growth (5×10^8 *Escherichia coli*/ml), and 50 μM-HOCl completely inhibited cell division (colony formation). These effects were apparent within 5 min of HOCl exposure, and were not reversed by extensive washings. DNA synthesis (incorporation of [^{3}H]-thymidine) was significantly affected by even a 1 min exposure to 50 μM-HOCl, and decreased by as much as 96% after 5 min. In contrast, bacterial membrane disruption and extensive protein degradation/fragmentation (release of acid-soluble counts from [^{3}H]leucine-labelled cells) were not observed at concentrations below 5 mM-HOCl. Protein synthesis (incorporation of [^{3}H]leucine) was only inhibited by 10–30% following 5 min exposure to 50 μM-HOCl, although longer exposure produced more marked reductions (80% after 30 min). Neutrophils deficient in myeloperoxidase cannot convert H_2O_2 to HOCl, yet can kill bacteria. We have found that H_2O_2 is only 6% as effective as HOCl in inhibiting *E. coli* growth and cell division (0.34 mM-H_2O_2 required for 50% inhibition of colony formation), and taurine or amino acids do not block this effect. Our results are consistent with a rapid and selective inhibition of bacterial cell division by HOCl in phagocytes. H_2O_2 may substitute for HOCl in myeloperoxidase deficiency, but by a different mechanism and at a greater metabolic cost.

INTRODUCTION

Phagocytic cells (neutrophils, monocytes, macrophages) play a primary role in the body's defence against bacterial infection [1–6]. Stimulated phagocytes engulf invading organisms and expose them to a series of oxygen radicals, related oxidants, and lytic enzymes. The primary oxygen radical generated by phagocytes is superoxide, which rapidly dismutates to form hydrogen peroxide (H_2O_2). Myeloperoxidase (MPO) then uses H_2O_2 to oxidize Cl^- ions to hypochlorous acid (HOCl) [4]. Although it is generally believed that oxidants such as HOCl and H_2O_2 function synergistically with lytic enzymes in bacterial killing, no clear role for these oxidants has been defined [2,3,5].

Both HOCl and H_2O_2 are bactericidal *in vitro* [1,6–16]. Since HOCl has a significantly higher oxidizing potential than H_2O_2 (E_0 for HCl is 1.63, whereas E_0 for H_2O_2 is 0.8 when considering one-electron reactions in protic media [17,18]), and is also a stronger germicidal agent [12,19] it is tempting to conclude that H_2O_2 (and superoxide) are generated merely as precursors of HOCl. Indeed, Klebanoff [10] has shown that MPO can potentiate the bactericidal activity of H_2O_2 *in vitro*. Interest in the role of HOCl *in vivo* was heightened by the work of Harrison & Schultz [4]. Using a system in which Millipore filters were impregnated with MPO, they were able to demonstrate oxidation of Cl^- by H_2O_2 and quantitate production of HOCl. A paradox is presented, however, by the description of a group of individuals who, while clinically not afflicted by serious bacterial infections, have phagocytes deficient in MPO [20–23]. Thus, questions remain about the participation of HOCl in bacterial killing *in vivo*.

HOCl is generally considered to be a highly destructive, non-selective oxidant which reacts avidly with all biomolecules. Observations that HOCl can oxidize nucleotides [24], activate latent enzymes [25], or inactivate enzymes [26,27] and electron transport systems [16], disrupt basement membranes [28] or cell membranes [29–31], and fragment proteins [32, J. M. S. Davies, S. M. McKenna, D. A. Horowitz & K. J. Davies, unpublished work] (The term protein fragmentation refers to the direct breakdown of proteins by oxidants. Such processes

Abbreviations used: MPO, myeloperoxidase.

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may involve both main-chain scission and side-chain scission by mechanisms which differ from peptide hydrolysis. In contrast, the term 'protein degradation' refers to peptide-bond hydrolysis by proteolytic enzymes) have led to proposals that these effects might underlie the bactericidal activity of HOCl. Importantly, however, Elsbach *et al.* [33] have shown that loss of cell division in neutrophil-ingested *Escherichia coli* occurs earlier than does inhibition of protein synthesis, and without concurrent protein degradation or membrane disruption. The image of HOCl as a non-selective, widely destructive agent appears to conflict, then, with the specific inhibition of bacterial cell division observed in phagocytes.

A specific oxidation of *E. coli* sulphydryls by HOCl was first reported by Thomas [14,15]. Rosen & Klebanoff later noted oxidation of bacterial iron-sulphur centres by the MPO–halide system preceding loss of cell viability [34]. The mechanism through which such oxidations contributed to cell death remained undetermined, however. More recently, Albrich *et al.* have suggested a specific attack of HOCl on the cell envelope, associated with loss of certain transport functions [35]. The series of experiments reported here test the ability of HOCl and H_2O_2 to inhibit bacterial growth selectively, with effects consistent with those observed in phagocytes. While comparing the relative potencies of the two oxidants as bactericidal agents, we evaluated their effects on cellular functions considered essential for growth and division.

A preliminary report of this work has been published [36].

MATERIALS AND METHODS

E. coli strain RM312, a prototrophic derivative of the W3110 strain of *E. coli* K-12 [37], was used in all experiments. *E. coli* were grown by overnight culture in M-9 minimal medium plus glucose (2 g/l), supplemented with 1 mM-$MgSO_4$ and 0.1 mM-$CaCl_2$ [38]. For each experiment, cells were subcultured in M-9 minimal medium plus glucose to exponential growth phase. Reagent grade NaOCl was obtained from Fisher Scientific. At the experimental pH of 7.4, NaOCl exists as a mixture of HOCl and OCl^-. We quantified NaOCl spectrophotometrically (292 nm) at pH 9.5 using an absorption coefficient of 350 $M^{-1} \cdot cm^{-1}$ (at this pH NaOCl exists almost exclusively as OCl^-). HOCl concentrations given in all Tables and Figures are actually the sum of HOCl + OCl^-.

E. coli survival was assessed both by colony formation on solid minimal (2% Bacto-agar), and by light scattering of cell suspensions at 595 nm. Colony formation was evaluated during a 72 h culture (37 °C) following treatment of *E. coli* (5×10^8 cells/ml) with HOCl or H_2O_2 (1–500 μM). Samples of 1 ml (in 16 mm × 100 mm glass test tubes) were incubated at 37 °C in a shaking water bath for 1 h in the presence of oxidant. Further growth was then inhibited by placing samples in an ice/water bath. After 1:10^5 dilution with cold medium, 0.1 ml aliquots of each sample were plated on agar. Colonies formed after each dose of oxidant, divided by the colonies formed by untreated samples, gave the survival fraction. A time course of oxidant effects on cell division was derived from changes in light-scattering properties. *E. coli* suspended at 10^8 cells/ml of M-9 minimal medium plus glucose (20 ml of *E. coli* suspension/125 ml Erlenmeyer flask) were treated with HOCl or H_2O_2 (50 nM–500 μM) and incubated at 37 °C in a shaking water bath. At intervals, 1 ml aliquots were withdrawn, placed in ice/water, and light scattering was compared with that of untreated samples for each time point. Changes in light-scattering properties were also used to evaluate the ability of amino acids and taurine to protect *E. coli* from the growth inhibition exerted by HOCl and H_2O_2. *E. coli* (2.5×10^8 cells/ml of M-9 minimal medium plus glucose) were incubated in a shaking water bath [37 °C for 90 min in the presence of 50 μM-HOCl or 500 μM-H_2O_2, ± amino acid (Ala, Cys, Lys, Met, Trp) or taurine, at a concentration 100–200-fold greater than that of the oxidant employed].

Incorporation of [^{3}H]leucine (into newly-synthesized proteins) was used as a measure of protein synthesis. *E. coli* (5×10^8 cells/ml) were treated with HOCl or H_2O_2 (37 °C for 1 h), then [^{3}H]leucine was added (0.09 μCi/ml final concn.). Protein synthesis was stopped after 1 h by addition of ice-cold trichloroacetic acid to a 10% final concentration. Precipitates were washed twice with 5% trichloroacetic acid, and dissolved in formic acid before measurement of acid-precipitable radioactivity.

The kinetics of HOCl inhibition of protein synthesis were also measured via incorporation of [^{3}H]leucine. *E. coli* [(2.5–5) × 10^8 cells in 1 ml of M-9 minimal medium plus glucose] were incubated at 37 °C in a shaking water bath for varying lengths of time with a single concentration of HOCl. At the end of each treatment period $Na_2S_2O_3$ was added to reduce unreacted HOCl. Protein synthesis was measured, as described above, by incorporation of [^{3}H]leucine (in acid-precipitable form) during a 1 h incubation.

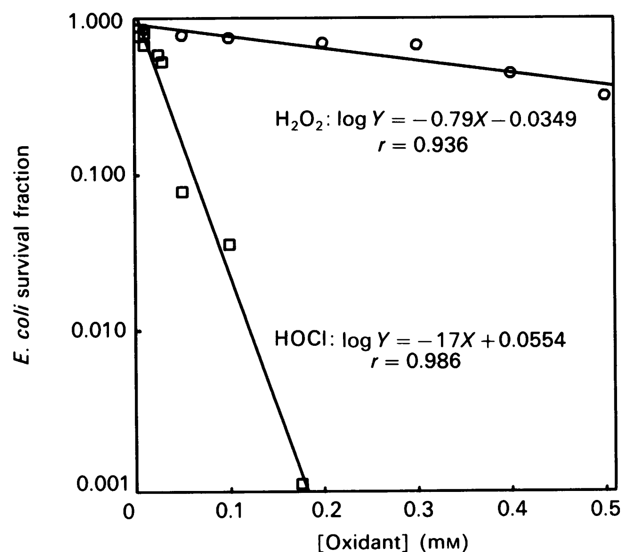

Fig. 1. Survival curves for *E. coli* treated with HOCl or H_2O_2

E. coli (5×10^8 cells/ml) were exposed to HOCl or H_2O_2 at the concentrations indicated. Treatments were performed at 37 °C for 1 h, in M-9 minimal medium plus glucose. Following 1:10^5 dilution with cold medium, 0.1 ml aliquots were plated on 2% Bacto-agar and cultured for 72 h at 37 °C. The survival fraction (colonies formed at each oxidant concentration/colonies formed by untreated samples) is plotted above on a logarithmic scale. Values are means of three independent determinations with S.E.M. < 10%.

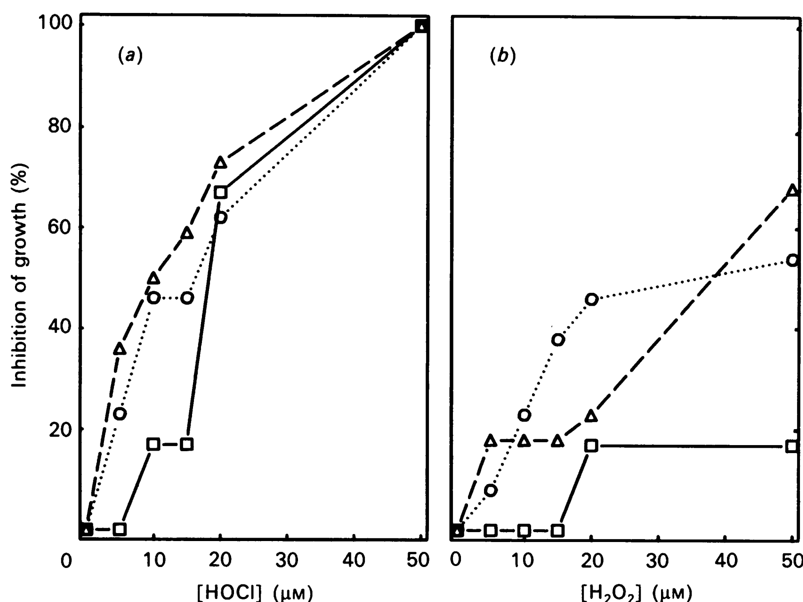

Fig. 2. Inhibition of cell growth by HOCl and H_2O_2

E. coli (10^8 cells/ml) were exposed to the concentrations of HOCl (*a*) or H_2O_2 (*b*) indicated for 5 min (□), 15 min (○) or 30 min (△). Treatments were performed at 37 °C, in M-9 minimal medium plus glucose. Cell growth was measured by light scattering at 595 nm. Values are means of three independent determinations with S.E.M. < 10%.

Protein degradation and membrane integrity were evaluated in pre-labelled cells. *E. coli* (5×10^8 cells/ml) were labelled by incorporation of [^{3}H]leucine (0.09 μCi/ml) in a pulse–chase procedure similar to that reported by Mosteller *et al.* [37]. Incorporation was conducted for 3 h (37 °C), after which cells were washed three times by centrifugation in M-9 minimal medium plus glucose with 75 μg of cold leucine/ml. Samples (1 ml) were treated with HOCl or H_2O_2 (37 °C for 1 h). Metabolism was stopped by placing samples in an ice–water bath.

Protein degradation was assessed by counting radioactivity released from cellular proteins in acid-soluble form after cell lysis with trichloroacetic acid. Water-soluble counts released by (centrifuged) intact cells, as well as the percentage of such counts which were also acid-soluble, served as indices of membrane disruption.

The effect of HOCl on DNA synthesis was studied in both RM312 and RM712, a thymidylate synthetase-deficient mutant derived from RM312. Experiments to determine the time course of DNA synthesis inhibition were similar to those described above for the study of protein synthesis inhibition. *E. coli* (5×10^8 cells/ml) were incubated with 50 μM-HOCl in M-9 minimal medium plus glucose. At the end of each treatment period 100 μM-Na_2SO_3 was added to quench any residual HOCl. Incorporation of [^{3}H]thymidine (5 μCi/ml) into acid-precipitable forms during a 90 min incubation was used as a measure of DNA synthesis. Total intracellular [^{3}H]thymidine was measured in cells washed twice by centrifugation and then solubilized with formic acid.

RESULTS

E. coli survival

HOCl had a dramatic effect on *E. coli* growth, as measured by inhibition of colony formation (Fig. 1). Following incubation with as little as 50 μM-HOCl, *E. coli* survival was only 7.85%, in marked contrast to 78.2% survival following the same dose of H_2O_2. Even after treatment with 500 μM-H_2O_2, 31.6% of the cells were still able to form colonies.

The inhibitory effect of HOCl on *E. coli* growth occurred rapidly (Fig. 2*a*). Measurements of the light-scattering properties of *E. coli* suspensions (595 nm) revealed 100% inhibition of growth by 50 μM-HOCl within 5 min. A lower cell concentration was used in Fig. 2 than that employed for the experiments of Fig. 1 (10^8 cells/ml instead of 5×10^8 cells/ml) in an attempt to maximize the bactericidal effect of H_2O_2. The difference in bactericidal potency of HOCl and H_2O_2 was again apparent, however, since 50 μM-H_2O_2 inhibited growth by < 20% within 5 min and < 70% within 30 min (Fig. 2*b*). The results in Fig. 2(*b*) present another, unexpected difference between the two oxidants. At concentrations of H_2O_2 below 50 μM, *E. coli* growth was inhibited less after 30 min treatment than after 15 min. This finding may suggest that *E. coli* can compensate for the damage exerted by low doses of H_2O_2. To verify that H_2O_2 was truly a weaker bactericidal agent than HOCl, and not just a slower acting one, incubation was extended (Fig. 3). Even after 180 min the inhibitory effect of H_2O_2 was not comparable with that of HOCl.

The growth inhibition seen in HOCl-treated cells was not reversible by 100-fold dilution or repeated washings (results not shown). Inclusion of taurine or certain amino acids during HOCl exposure, however, protected *E. coli* from the effects of HOCl (Table 1). Cells treated with 50 μM-HOCl in the presence of 10 mM-alanine, lysine, methionine, tryptophan or taurine continued to grow to 80–100% of control levels. The lack of protection by cysteine has not been explored. Even at concentrations of 100 mM, none of the amino acids employed, nor taurine, protected *E. coli* from the growth inhibition effected by 500 μM-H_2O_2.

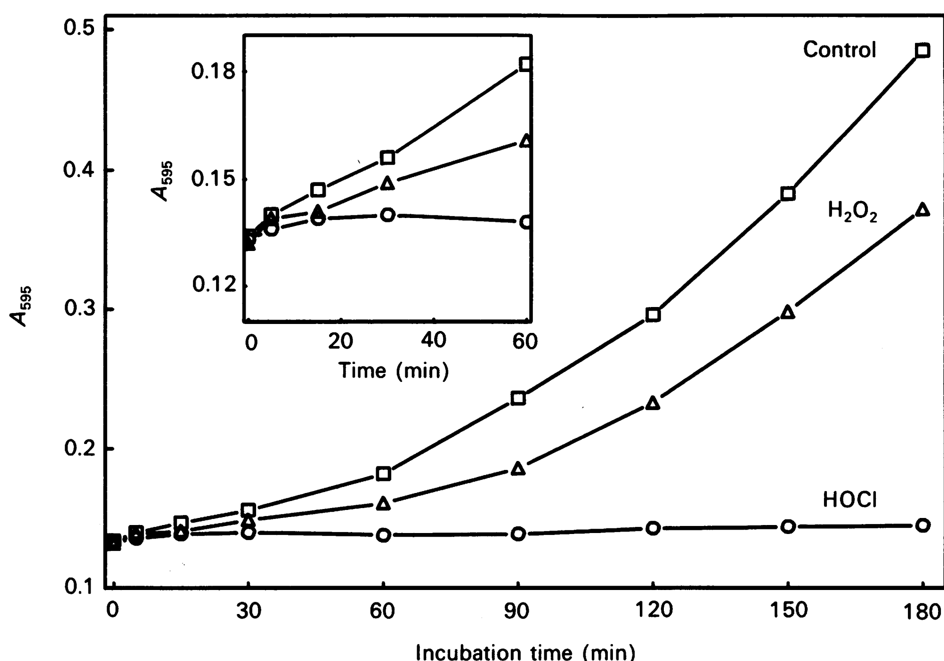

Fig. 3. Time course of growth inhibition by HOCl and H_2O_2

E. coli were exposed to 20 μM-HOCl or H_2O_2 (or were untreated) as described in the legend to Fig. 2. Cell growth, over a 3 h period, is represented by light scattering (absorbance) measurements at 595 nm. The inset repeats the results from 0–60 min on an expanded scale. Values are means of three independent determinations with S.E.M. < 10%.

Table 1. Amino acids and taurine protect *E. coli* against the growth inhibition exerted by HOCl

E. coli (2.5×10^8 cells/ml) were grown at 37 °C for 90 min in M-9 minimal medium plus glucose. Where used, 50 μM-HOCl, 10 mM-amino acid or 10 mM-taurine were present throughout the entire 90 min incubation. Increases in A_{595} (light scattering at 595 nm) were measured as a reflection of cell growth. Values are means of three independent determinations with S.E.M. < 10%.

Additions	Increase in A_{595}	Growth (% of normal growth)
None	0.213	100
HOCl	−0.036	0
HOCl + alanine	0.186	87
HOCl + cysteine	−0.019	0
HOCl + lysine	0.218	102
HOCl + methionine	0.200	94
HOCl + tryptophan	0.221	104
HOCl + taurine	0.212	99

Protein synthesis

Following a 1 h incubation, incorporation of [^{3}H]-leucine into newly-synthesized bacterial proteins was effectively inhibited by HOCl (Fig. 4). Again, the potency of HOCl was several-fold greater than that of H_2O_2. From our results (Fig. 5), however, it appears that inhibition of protein synthesis may lag behind inhibition of cell growth (Figs. 2 and 3). Protein synthesis was inhibited by 10% within 5 min, in contrast to 100% inhibition of growth by 50 μM-HOCl within 5 min (Fig. 2*a*). The inhibitory effect on protein synthesis was maximal only after 20 min exposure to HOCl.

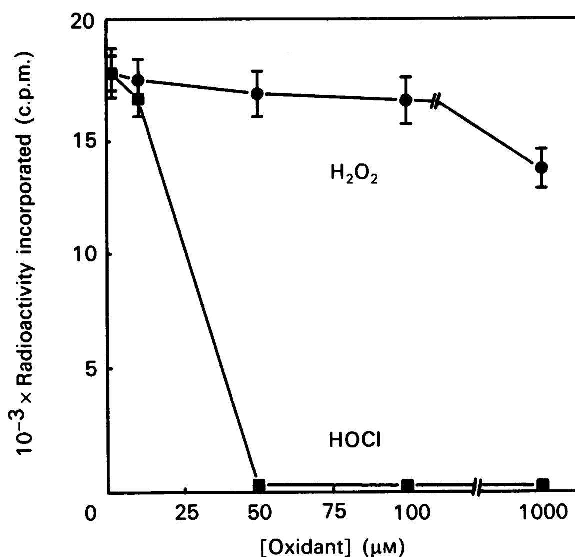

Fig. 4. Inhibition of protein synthesis by HOCl and H_2O_2

Protein synthesis was assessed by incorporation of [^{3}H]-leucine into acid-precipitable forms (see the Materials and methods section) following a 1 h incubation. *E. coli* (2×10^8 cells/ml) were incubated at 37 °C in M-9 minimal medium plus glucose. Values are means ± S.E.M. of three independent determinations.

Cell membrane integrity and protein fragmentation/degradation

Light scattering (as a measure of cell growth) did not decrease after treatment of *E. coli* suspensions with 50 μM-HOCl (Figs. 2 and 3), indicating that cell death occurred without gross disruption of the membrane.

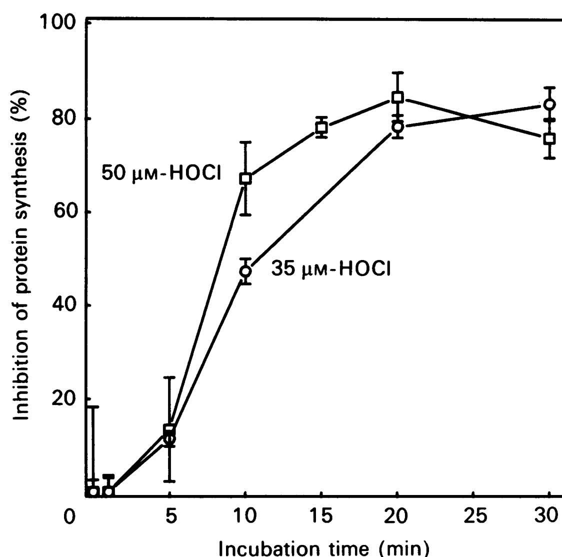

Fig. 5. Time course of the inhibition of protein synthesis by HOCl

Protein synthesis was measured by incorporation of [^{3}H]-leucine into acid-precipitable forms, as described in the legend to Fig. 4. The symbols represent 50 μM-HOCl with 5×10^8 *E. coli*/ml (□) and 35 μM-HOCl with 2.5×10^8 *E. coli* (○). Values are means ± S.E.M. of three independent determinations.

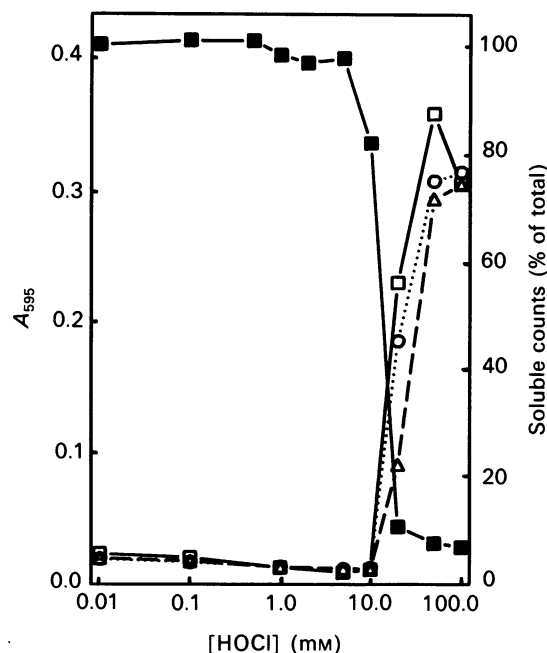

Fig. 6. Effects of HOCl on membrane integrity, protein fragmentation and protein degradation

E. coli (5×10^8 cells/ml) were incubated with HOCl for 1 h at 37 °C. Light scattering (absorbance at 595 nm) was measured as a direct index of membrane disruption (■——■). In parallel experiments cells were pre-labelled by incorporation of [^{3}H]leucine (see the Materials and methods section). Water-soluble counts released from cells, following centrifugation at 1500 *g*, were measured by liquid scintillation (□). The proportion of water-soluble counts which were also acid-soluble was determined by treatment of the 1500 *g* supernatants with 10% trichloroacetic acid (○·····○). Finally, aliquots of pre-labelled cells were directly precipitated with 10% trichloroacetic acid, and the total acid-soluble counts were measured (△). Values are means of three independent determinations with S.E.M. < 10%.

These data, in conjunction with studies using pre-labelled *E. coli* (Fig. 6), indicate that low concentrations of HOCl (although bactericidal) cause neither significant membrane disruption nor extensive protein breakdown. Only 10 mM-HOCl or greater led to decreased light scattering, suggesting that membranes were intact until that point. Similarly, radioactivity was not released from *E. coli* whose proteins had been prelabelled with [^{3}H]leucine unless the concentration of HOCl added was 10 mM or higher. The majority of the counts released in water-soluble forms were also present in acid-soluble forms, indicating that released proteins were fragmented ($M_r \leqslant 5000$). Previous work has shown that certain oxidants (e.g. the hydroxyl radical) can increase the susceptibility of cell proteins to proteolytic digestion [39–45]. Intracellular proteases may have contributed to the release of acid-soluble counts in the present experiments (i.e. true protein degradation). Independent experiments have shown, however, that the proteolytic capacity of *E. coli* is insufficient to explain the extensive protein breakdown observed at high concentrations of HOCl [39,42,44,45].

Similar experiments with H_2O_2 also indicate that membrane leakage, protein fragmentation and protein degradation cannot explain the bactericidal activity observed with this oxidant. Low concentrations of H_2O_2 do increase protein degradation in *E. coli* to approximately twice the basal rate with 1 mM-H_2O_2 [39,42,44,45]. Higher concentrations of H_2O_2, however, produce little or no extra stimulation of intracellular proteolysis [44]. We also have not observed fragmentation of *E. coli* proteins or gross loss of membrane integrity at bactericidal concentrations of H_2O_2.

DNA synthesis

Incorporation of [^{3}H]thymidine (during a 90 min incubation) into newly-synthesized DNA was significantly inhibited after brief exposure of bacteria to 50 μM-HOCl (Fig. 7). DNA synthesis in RM312 decreased by at least 48% after only 1 min treatment with HOCl, and by as much as 96% after 5 min exposure. Inhibition of DNA synthesis in RM712 (the thymidylate synthetase-deficient mutant) was also strongly, and rapidly, inhibited by 50 μM-HOCl. These results suggest that inhibition of DNA synthesis may play an important role in the inhibition of cell division by HOCl.

Total intracellular [^{3}H]thymidine in untreated *E. coli* averaged 41800 c.p.m., compared with 21600 c.p.m. incorporated into acid-soluble counts. In a series of experiments, total counts ranged from 1.5–2.8-fold higher than incorporated counts. Although total counts in HOCl-treated samples decreased by as much as 40% after 1 min and 70% after 5 min treatment, the ratio of total counts to incorporated counts actually increased. Samples treated for 1 min had 1.8–4.6-fold more intracellular [^{3}H]thymidine than was incorporated into DNA. Treatment for 5 min increased that ratio even further in 3–20-fold higher intracellular radioactivity than that incorporated. Thus, it seems reasonable to suggest that thymidine uptake did not limit thymidine incorporation.

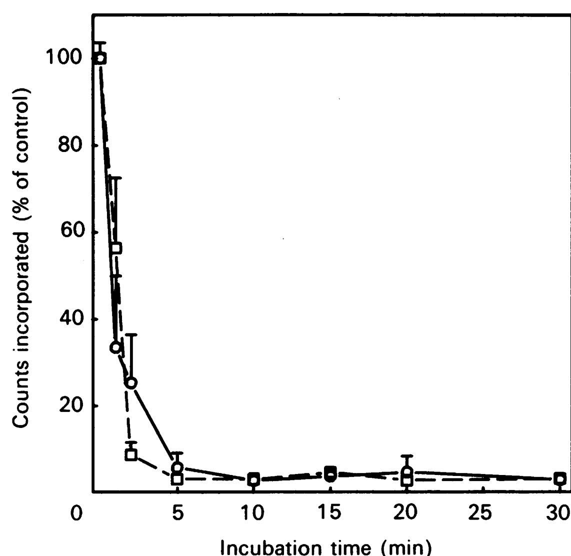

Fig. 7. Effects of HOCl on DNA synthesis

E. coli (5×10^8 cells/ml) were treated with 50 μM-HOCl at 37 °C. After addition of $Na_2S_2O_3$ (to quench any remaining HOCl) incubation was continued for 2 h in the presence of 5 μCi/ml [^{3}H]thymidine. DNA synthesis was measured by incorporation of [^{3}H]thymidine into acid-precipitable forms in RM312 (○) or in a thymidylate synthetase-deficient mutant, RM712 (□). Values are means ± S.E.M. of three independent determinations.

Table 2. Inhibition of *E. coli* cell growth, protein synthesis, and DNA synthesis after incubation with HOCl

HOCl (50 μM) was added to a single (10 ml) sample of *E. coli* (5×10^8/ml) in M-9 medium plus glucose. After 2 min or 5 min incubation at 37 °C, $Na_2S_2O_3$ (100 μM) was added to quench residual HOCl. Three aliquots were then removed and analysed respectively for colony formation, leucine incorporation and thymidine incorporation (as described under Materials and methods). Each measurement was made in triplicate samples. The results above are the means ± S.E.M. of three independent experiments.

Function	Incubation time with HOCl ...	Inhibition (%) 2 min	Inhibition (%) 5 min
Colony formation		33 ± 3	65 ± 3
[^{3}H]Leucine incorporation		5 ± 4	36 ± 9
[^{3}H]Thymidine incorporation		61 ± 10	66 ± 5

Simultaneous measurements of cell growth, protein synthesis and DNA synthesis

In the experiments above, functions essential for cell growth were studied independently. Simultaneous evaluation of these functions in the detail desired was not experimentally feasible. To confirm our results, however, a more limited experiment was performed to assess colony formation, protein synthesis and DNA synthesis after exposure of a single sample to 50 μM-HOCl. The results presented in Table 2 are consistent with earlier findings.

DISCUSSION

Stimulation of phagocytes by bacteria leads to an increased O_2 consumption and release of superoxide and related products of oxygen reduction [1–3]. The action of these oxidants, in conjunction with the lytic enzymes released from granules, culminates in bacterial death. Studies of patients with chronic granulomatous disease emphasize the importance of oxygen metabolites in bacterial killing. Phagocytes from these patients, which are unable to form superoxide and subsequent oxidants, cannot kill catalase-positive bacteria [1–33]. The mechanism by which the reactive oxygen products kill bacteria has not previously been determined [2,3,5]. The present results suggest that phagocytes may utilize HOCl as a bacteriostatic agent. The bacteriostatic effect of HOCl may derive from the ability of this oxidant to inhibit DNA synthesis. Partial inhibition of protein synthesis by HOCl may also be important in decreased bacterial growth and division.

The bactericidal activity of H_2O_2 *in vitro* is clearly potentiated by MPO plus a halide [10]. Interest in the bactericidal properties of HOCl was stimulated by Harrison & Schultz's demonstration that myeloperoxidase catalyses the oxidation of chloride ions; $H_2O_2 + Cl^- \xrightarrow{MPO} HOCl$ [4]. Our finding that 50 μM-HOCl is highly bactericidal echoes the results of McRipley & Sbarra [9] and Klebanoff [10] in which the addition of a lysate of neutrophil granules, or MPO, increased the bactericidal activity of 50 μM-H_2O_2 to 99 %. The present results indicate that low concentrations of HOCl can exert a selective inhibition of bacterial growth and division. No gross structural damage was associated with this bactericidal activity, and intracellular protein was not appreciably degraded or fragmented. Even protein synthesis appeared to be inhibited indirectly. Our data do not allow, however, complete discrimination between a primary inhibition of protein synthesis and an indirect effect following inhibition of cell division. Other oxidants have been shown to inhibit protein synthesis [39]. While HOCl inhibited *E. coli* protein synthesis by only 10 % within 5 min, it is conceivable that the proteins involved are crucial for cell growth, and thus represent primary targets.

DNA synthesis, in contrast to the apparent gradual inhibition of protein synthesis, was quickly interrupted. A marked decrease in DNA synthesis was seen after only 1 min of treatment of *E. coli* suspensions with 50 μM-HOCl. By 5 min, DNA synthesis in RM312 decreased as much as 96 %. DNA synthesis in RM712, a thymidylate synthetase-deficient mutant dependent on exogenous supplies of thymidine, was also quickly inhibited. The similarity of results obtained with RM712 and RM312 suggests that thymine salvage pathways were not utilized in the wild-type derivative. It should be noted that $Na_2S_2O_3$ was added to HOCl-treated cells before incubation with [^{3}H]thymidine. Therefore, the inhibition of DNA synthesis reported in Fig. 7 cannot be due to modification of [^{3}H]thymidine by HOCl ($Na_2S_2O_3$ alone had no effect). Furthermore, measurements of [^{3}H]-thymidine uptake indicate that impaired membrane transport of [^{3}H]thymidine may not explain the inhibition of DNA synthesis exerted by HOCl.

Our results are consistent with the observations of Elsbach *et al.* [33]. Their studies showed no significant damage to the structural integrity of *E. coli* membranes

after phagocytosis and, even though cell division ceased, protein synthesis continued for at least 1 h. The work of Albrich *et al.* [35] also concludes that the bactericidal effect of HOCl on *E. coli* is not linked to changes in membrane permeability. They report, however, that uptake of [^{14}C]leucine decreases following exposure of cells to HOCl. This would appear to conflict with the observations of Elsbach *et al.* and the current studies, in which incorporation of [^{3}H]leucine into newly-synthesized protein proceeded for 30–60 min after growth inhibition. It may be, however, that the transport function remaining (10–40% in the results of Albrich *et al.*) is sufficient for protein synthesis to continue.

An incidental finding that many of the *E. coli* colonies which survived HOCl treatment were smaller than control colonies is unexplained. Hassan & Fridovich [46], studying damage induced by paraquat, and Passo & Weiss [11], reporting oxidative damage by phorbol myristate acetate-stimulated neutrophils, have noted growth of surviving *E. coli* in 'microcolonies' [11]. Whether the events that impede bacterial division are related to this change in colony size has not been explored.

Identification of subjects deficient in MPO, but clinically well, led some [20,46] to question the role of the MPO/halide system in bacterial killing *in vivo*. As discussed by Test & Weiss [5], several investigators have reported prolonged H_2O_2 production in MPO-deficient neutrophils [21–23, 48–51]. Our results indicate that amounts of H_2O_2 which might accumulate during such prolonged periods are bactericidal (Fig. 2*b*). Thus, H_2O_2 may substitute for HOCl at a greater metabolic cost [3,21]. Additionally, other potentially lethal phagocyte products (granular proteins) may have enhanced roles in the bacterial killing by MPO-deficient cells [5,6].

Although direct determination of HOCl concentrations in phagocytes has not, so far, been possible, indirect measurements of HOCl (as well as superoxide and H_2O_2) production or release have been published by several investigators [1,5,8–10,52]. From such studies it would seem that 50 μM-HOCl, which was highly bacteristastic in this investigation, is a realistically attainable physiological concentration. Our experiments with purified HOCl may provide a model which closely resembles known parameters of early bactericidal events within phagocytes. The results are consistent with a primary role for HOCl in bacterial killing by phagocytes. Other data suggest that HOCl may have a rapid inhibitory effect on RNA synthesis as well as DNA synthesis [53]. HOCl may react with bacterial membrane components which are essential for cell division. HOCl may disrupt membrane/DNA interactions needed for replication, alter the DNA template itself, or inactivate enzymes of the replication system. Alternatively, HOCl may inhibit synthesis of critical proteins required for DNA replication and/or cell division. Such reactions may be rapid and quite selective. Once the capacity for cell division has been lost, the lytic enzymes of phagocytes (proteinases, lipases, etc.) may degrade the dead cells at a more leisurely pace.

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