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**Research Article**

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# Effects of the Putative Neutrophil-generated Toxin, Hypochlorous Acid, on Membrane Permeability and Transport Systems of *Escherichia coli*

**J. Michael Albrich, James H. Gilbaugh III, Kerry B. Callahan, and James K. Hurst**
*Department of Chemical, Biological, and Environmental Sciences, Oregon Graduate Center, Beaverton, Oregon 97006-1999*

## Abstract

**Titrimetric addition of hypochlorous acid (HOCl) or chloramine ($NH_2Cl$) to suspensions of *Escherichia coli* decreases their ability to accumulate $^{14}C$-labeled glutamine, proline, thiomethylgalactoside, and leucine in a manner that approximately coincides with loss of cell viability; quantitative differences in cellular response are observed with the two oxidants. Inhibition of β-galactosidase activity in *E. coli* ML-35, a strain lacking functional lactose permease, is complex and also depends upon the identity of the oxidant. Membrane proton conductivities and glycerol permeabilities are unchanged by addition of HOCl or $NH_2Cl$ in excess of that required for inactivation. The combined results are interpreted to indicate that the locus of HOCl attack is the cell envelope, that HOCl inactivation does not occur by loss of membrane structural integrity, that loss of transport function can be identified with either selective oxidative inhibition of the transport proteins or loss of cellular metabolic energy, and that different mechanisms of inactivation may exist for HOCl and $NH_2Cl$.**

## Introduction

Hypochlorous acid (HOCl)[1] is a potent microbicide generated in stimulated neutrophils by myeloperoxidase (MPO)-catalyzed peroxidation of chloride ion (1–8). The mechanism of bactericidal action, however, is not understood (9). Investigation by a number of laboratories has suggested that the bacterial membrane is the site of lethal attack by HOCl. Friberg (10) demonstrated that *Escherichia coli* grown on $^{32}P$ leaked 10–15% of this label on treatment with HOCl and suggested that cell membrane permeability had been altered. Venkobachar et al. (11) detected both RNA and protein in supernatants of centrifuged *E. coli* suspensions reacted with bactericidal concentrations of HOCl, which they attributed to a membrane permeability change. Camper and McFeters (12) found that uptake of $^{14}C$-labeled glucose and algal protein hydrolysate was reduced after HOCl treatment. Because they could demonstrate that aldolase, a sulfhydryl enzyme inactivated by HOCl (13), retained full activity in organisms exposed to bactericidal concentrations of HOCl, they concluded that the lethal reactions occur before HOCl reaches the cytoplasm, e.g., at the membrane. Sips and Hamers (14) observed that an *E. coli* mutant, ML-35 ($lac\ i^-\ z^+\ y^-$), treated with HOCl, a cell-free MPO-$H_2O_2$-$Cl^-$ system, or leukocytes increased its rate of hydrolysis of *o*-nitrophenylgalactoside (ONPG). This mutant has no functional lactose permease capable of transporting the galactoside into the cytoplasm, but does possess normal levels of cytosolic β-galactosidase. Since these researchers could detect no β-galactosidase activity in supernatants of centrifuged HOCl-treated bacterial suspensions, they concluded that rise in ONPG hydrolysis (and killing) was due to disruption of the membrane permeability barrier.

Results of our survey of the reactivity of HOCl with prototypic bacterial cellular components (13) suggest that membrane proteins possessing nucleophilic sites are susceptible to oxidation but that oxidative disruption of the cellular envelope is not expected. Consistent with this view, no ultrastructural changes are observed by electron microscopy in HOCl-treated *E. coli* (15). This does not exclude the possibility of membrane permeability changes, which might allow leakage of small molecules, i.e., electrolytes and metabolites. Thus, we have investigated two properties of HOCl-treated *E. coli* which reflect cytoplasmic membrane integrity toward small molecules: $H^+$-conductance (16) and glycerol permeability (17, 18). To test the hypothesis that susceptible membrane proteins may be altered by HOCl oxidation, we have investigated uptake of radiolabeled substrates for a variety of membrane transport systems. This study encompasses both ATP-driven (glutamine, leucine) and proton-motive force-driven (thiomethylgalactoside [TMG], proline) transport systems, which differ both in modes of energization and structural organization (19).

Chloramine ($NH_2Cl$), produced by the action of neutrophil-generated HOCl on ammonium ion, has recently been suggested as a bactericidal and cytotoxic agent (2, 3). In addition, a variety of *N*-chloro compounds are known to be generated by treating bacteria with HOCl or the MPO-$H_2O_2$-$Cl^-$ systems (20). Therefore, we have also investigated the effect of $NH_2Cl$ upon transport.

## Methods

### *Reagents*

Hypochlorous acid solutions were prepared and analyzed according to previously described procedures (13, 21). Chloramine solutions were prepared by adding stock HOCl to a buffer solution containing 154 mM NaCl, 100 mM phosphate, pH 7.4, and 100 mM $NH_4Cl$. This concentration of $NH_4Cl$ was a 20-fold excess over the maximum amount of HOCl added (5 mM). The resultant solution was allowed to stand 5 min. The final concentration of $NH_2Cl$ was taken to be identical to the concentration of added HOCl (22). Radiolabeled ($^{14}C$-U)glutamine, ($^{14}C$-methyl)TMG, ($^{14}C$-U)proline, and ($^{14}C$-U)leucine were obtained from New England Nuclear, Boston, MA. Other chemicals and biochemicals were the best available quality purchased from commercial suppliers.

Address correspondence to Dr. Hurst.
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1. *Abbreviations used in this paper:* HOCl, hydrochlorous acid; MPO, myeloperoxidase; ONPG, *o*-nitrophenylgalactoside; TDG, thiodigalactoside; TMG, thiomethylgalactoside.

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### *Microorganisms*

*E. coli* strain ATCC 25922 was obtained from the Microbiology Laboratory, Good Samaritan Hospital, Portland, OR; strain ML-35 (lac $i^-$ $z^+$ $y^-$) was the kind gift of Dr. T. Hastings Wilson, Harvard University, Cambridge, MA. Bacterial cell concentrations were routinely measured spectrophotometrically after calibration by pour-plate quantitative culture; an absorbance per centimeter of 1.0 at 540 nm corresponds to 3–5 × $10^8$ colony-forming U/ml and 0.33 mg *E. coli* dry weight. For proton conductance and glycerol permeability experiments, both strains were grown aerobically with shaking on minimal media, plus either 0.5% glucose or 0.4% glycerol as carbon source, then harvested in late log growth phase by centrifugation, washed twice at 4°C using a buffer appropriate to the particular study, and suspended in buffer to an absorbance/cm of 1.0–6.0 at 540 nM and kept on ice; bacteria so prepared were used within 4 h. For transport studies, *E. coli* 25922 was grown overnight aerobically at 37°C in 50 ml of nutrient broth (Difco Laboratories, Inc., Detroit, MI), 8 g/liter. This suspension was used as inoculum for 1 liter of the same medium and allowed to incubate for 3.5 h under the same conditions until harvested in late log phase. In studies involving transport by the lactose permease system in *E. coli* 25922, 0.5 mM isopropyl-β-D-thiogalactoside was added to the growth medium to induce the lac operon. Late log growth phase was chosen for cell collection because the permease activity is optimal during this period (23).

Immediately before use, aliquots of cell suspensions were transferred to an open vial, aerated with a stream of oxygen, and placed in a combination shaker-water bath at 23°C for 6 min. Cell suspensions were then flow-mixed with HOCl or $NH_2Cl$ solutions of varying concentrations, followed by quenching of excess oxidant with sodium thiosulfate ($Na_2S_2O_3$). In initial studies, the three-syringe flow-mixer configuration previously described (21) was used to provide rapid quenching; the procedure of manual addition of thiosulfate solutions was adopted in subsequent work since, with HOCl, we found no difference in viable cell counts by the two methods. Killing was time-dependent within this domain for $NH_2Cl$ (Fig. 7); in this instance, $Na_2S_2O_3$ was either flow-mixed with the *E. coli*-$NH_2Cl$ solution or added 5 min later. A separate aliquot of the *E. coli* stock suspensions was used for each measurement. In each experiment, 10 ml of cell suspension was mixed with 0.5 ml HOCl; to avoid mixing artifacts, the first 2–3 ml of flow-mixed effluent was discarded and collection was stopped just before complete evacuation of the drive syringes. A 0.1-ml aliquot of the product solution was taken for pour-plate culture analysis and the remainder was taken for measurement of membrane functional capabilities.

### *Membrane permeabilities*

*Proton conductance.* Proton conductance was measured by pH-jump perturbation (24). The method involves suspending a sufficient number of metabolically poisoned cells in a weakly buffered medium such that the internal buffer capacity of the cells is a significant fraction of the total buffer capacity. Small perturbations caused by strong acid or base addition are followed by a slower relaxation toward equilibrium values that are closer to the initial pH of the system; this relaxation is attributed to equilibration of the protons within the intracellular compartment with the external medium. The proton conductance, $C_m^{H+}$, is given by the equation: $C_m^{H+} = (0.7\ B_0 B_i / B_T)/t_{1/2}$, where $t_{1/2}$ is the relaxation half-time and $B_0$, $B_i$, and $B_T$ are extracellular, intracellular, and total buffer capacities determined from the amplitudes of the pH perturbation and relaxation; $C_m^{H+}$ is usually expressed in units of microgram ions $H^+$/s per pH unit per gram, dry weight, of bacteria.

Bacteria were suspended to 2–4 × $10^9$ cells/ml in 0.1 M KCl. After HOCl treatment and $Na_2S_2O_3$ quenching, 7.4 ml of the cell suspension was placed in an 18-ml vial, followed by 0.4 ml 1 M KSCN, 0.2 ml 80 mM 2-deoxyglucose, 20 μl 8 mM valinomycin in ethanol, and 20 μl 10 mg/ml carbonic anhydrase. It is important to use fresh, i.e., active, solutions of carbonic anhydrase to avoid artifactual relaxations arising from $CO_2$-$HCO_3^-$ equilibration (24). In investigations of valinomycin-dependent potassium transport in *E. coli*, EDTA ion is often added to improve accessibility of the ionophore to the bacterial plasma membrane (25); EDTA was not used in this study because it also enhances the nonselective membrane permeability to small molecules in this organism (26). Although valinomycin was added to provide some $K^+$ transport capability, the permeant anion, $SCN^-$, was also provided at sufficiently high concentration levels (50 mM) to ameliorate charge effects across the membrane which might impede proton movement (24). Carbonyl cyanide *m*-chlorophenylhydrazone, 20 μl of a 16 mM ethanol solution, was also added to the suspensions for studies requiring a proton ionophore.

A combination pH electrode (Orion Scientific Instruments Corp., Pleasantville, NY) was placed in the vial through a serum cap which snugly fitted both electrode and vial. The solution was magnetically stirred and deoxygenated with a stream of water-saturated nitrogen gas for a minimum of 10 min at 23°C. The vial and electrode were enclosed in a glove bag which was also degassed with nitrogen. The solution pH was measured using an Orion Ionnalyzer (710A; Orion Scientific Instruments Corp.); time-dependent changes were measured with an attached Linear Model 355 strip chart recorder. The initial acidity was adjusted to give equilibrium values of 7.0±0.2 pH units. By addition of deoxygenated solutions of 10 mM HCl or KOH, perturbations of 0.10–0.15 pH unit were made and the response of the system was recorded. Repetitive perturbations were made on a single sample, with sufficient time being given for equilibration between measurements. The relaxational response was first-order over the range of at least three half-lives.

*Glycerol permeability.* Addition of glycerol to suspensions of *E. coli* causes an immediate turbidity increase arising from dehydration of the cells in response to the increased osmotic pressure of the external medium (17). The turbidity then undergoes a slow exponential decline as glycerol (and water) enter the cell. Ultimately, an equilibrium state is reached, which is characterized by a turbidity that is less than the initial value by the amount of dilution caused by the added glycerol. The relaxation time constant is taken to be the reciprocal of the rate constant for diffusion of glycerol across the bacterial cytoplasmic membrane (17, 18).

Since glucose-grown *E. coli* are relatively impermeable to glycerol (18), bacteria grown on this carbon source were used for the permeability studies. Harvested bacteria were suspended at 5 × $10^8$ cells/ml in 25 mM sodium phosphate, pH 7.4, containing 0.1 M NaCl. After treatment with HOCl, 2.5 ml of this suspension was mixed rapidly with 0.5 ml 4.8 M aqueous glycerol in a 1-cm path-length optical cell and placed in the sample beam of a spectrophotometer (Model 16, Cary Instruments, Monrovia, CA); 2.5 ml of the same suspension and 0.5 ml of buffer were placed in the reference beam. This procedure requires ~15 s. Turbidity changes were monitored at 540 nm and displayed on a strip chart recorder (G-2000; Varian Associates, Inc., Palo Alto, CA).

### *Transport substrate uptake studies in E. coli 25922*

For experiments with $^{14}C$-radiolabeled compounds, sufficient substrate (6–10 μM) was added to 1.0-ml aliquots of 6 × $10^8$ *E. coli*/ml in 100 mM phosphate, pH 7.4, to give 0.5 μCi/ml radioactivity. The suspensions were incubated for 2.0 min at 23°C, at which point the reaction was terminated by rapid filtration on 0.45 μm cellulose triacetate membrane filters (GA-6; Gelman Sciences, Inc., Ann Arbor, MI). The filters were washed once with 4.0 ml buffer at 23°C, air dried, placed in vials containing 6.0 ml Aquasol-2 (New England Nuclear), and counted in a scintillation counter (Model LS-3133P; Beckman Instruments, Inc., Fullerton, CA). Uptake is determined from counts per minute less background. Background radioactivity was taken to be the zero-time extrapolation of measured time-dependent $^{14}C$-metabolite uptake curves determined for either untreated or HOCl-oxidized *E. coli*. Differences between background levels in oxidized and untreated bacteria were not significant. Values obtained ranged from 1,000 to 3,500 cpm, which is <12% of the counts for untreated bacteria after 2.0 min of incubation.

### *ONPG hydrolysis in E. coli ML-35*

Enzymatic hydrolysis of ONPG by HOCl-treated *E. coli* was measured both for intact bacteria and for cell homogenates. Organisms to be lysed were diluted to 6–8 × $10^8$ cells/ml, cooled to 0°C in an ice bath, and purged of oxygen by bubbling nitrogen through the suspension. Lysis

was accomplished by ultrasonic dispersal for 3 min in ice using the microtip probe of a sonicator cell disruptor (model W185F; Heat Systems-Ultrasonics, Inc., Farmingdale, NY) operated at 35 W; nitrogen was blown across the solution surface during sonication to minimize exposure to oxygen. The enzymatic assay was made by adding 60 $\mu$l 50 mM ONPG to 3.0 ml *E. coli* suspension in a 1.0-cm path-length optical cell. Appearance of the *o*-nitrophenolate ion was monitored spectrophotometrically at 420 nm; rates were calculated from the initial slopes of the traces using $\epsilon_{420} = 7.5\ mM^{-1}\ cm^{-1}$ (14). To determine intracellular and extracellular location of ONPG hydrolytic activity, ML-35 suspensions were centrifuged at 13,000 *g* for 2 min and supernatant and whole cell assays were compared.

## Results

*Proton permeabilities.* Proton conductances for *E. coli* strains 25922 and ML-35 determined from the equation given in Methods are plotted as a function of increasing HOCl oxidant levels in Fig. 1 *A* and *B*, respectively. The data presented are for experiments performed on single isolated cultures; comparison is made with cell viability determined from aliquots of the same suspensions upon which the proton conductances were measured. This procedure was adopted because the amount of HOCl required to inactivate the cells varied by as much as 30% from culture to culture, precluding quantitative comparison of separate analyses on different bacterial suspensions. Direct comparison of physical properties with cell viabilities measured on individual cultures always gave reproducible correlations, however. Measured $H^+$-conductances were identical regardless of whether the initial perturbation involved acidification or alkalinization, and were unchanged over several repetitive cycles. Within the experimental uncertainty of the measurements, proton conductances are unchanged by addition of HOCl well in excess of that required to sterilize the suspensions. A two- to threefold variation in $C_m^{H+}$ is routinely observed in this type of measurement (16). The apparent 2.5-fold increase in conductance upon addition of a lethal dose of HOCl to ML-35 (Fig. 1 *B*) may therefore be artifactual; it was not consistently observed in studies on various growth cultures. In contrast, addition of the proton ionophore, CCCP, to the suspensions caused loss of the relaxational step; the magnitude of the pH change in these systems corresponded to the total buffer capacities ($B_T$) in the unmodified suspensions, indicating that rapid equilibration of intracellular and extracellular compartments had taken place. Correspondingly, $C_m^{H+}$ must have increased by at least 10-fold, the lower limit being set by the response time of the pH electrode. The internal buffer capacity ($B_i$) for both ML-35 and ATCC 25922 varied from 40 to 50 $\mu$g ion $H^+/\Delta pH$-g dry weight *E. coli*. Total buffer capacity ($B_T$) was 90±10 $\mu$g ion $H^+/\Delta pH$-g dry weight *E. coli* for both organisms, which is less than that obtained for *Streptococcus lactis* (~130 $\mu$g ion $H^+/\Delta pH$-g bacteria) (16), but very similar to values found in mitochondria (27). Proton conductances measured for these cells (Fig. 1) are nearly identical to previously determined values for *S. lactis*, i.e., $C_m^{H+}$ = 0.1–0.3 $\mu$g ion H+/s · ΔpH · g bacteria (16).

*Glycerol permeabilities.* For both *E. coli* strains grown on glucose-minimal media, only small changes in rates of swelling of glycerol-plasmolyzed cells were observed upon treatment with HOCl to concentration levels well in excess of that necessary for culture sterilization. Permeabilities measured for *E. coli* ML-35, expressed as the reciprocal of the half-time for first-order swelling, are plotted against HOCl concentration levels in Fig. 2; cell viabilities measured on aliquots of the same samples used for the permeability studies are given for comparison. The experiments described in Fig. 2 were made on a single growth culture; comparable values were obtained with other ML-35 cultures and with ATCC 25922 cultures, although in the latter strain the value of $(t_{1/2})^{-1} = 0.04\ s^{-1}$, indicating slightly less intrinsic membrane permeability to glycerol. These rate constants compare favorably with previously reported values (18, 28). When *E. coli* 25922 was grown on glycerol, which is known to induce a membrane pore with broad specificity for *vic*-diols (28), no optical changes are seen on the timescale of observation when measured against a buffer diluted HOCl-treated *E. coli* suspension as reference. At all levels of HOCl oxidation investigated, rates of glycerol entry are too rapid to measure by the method employed.

In addition to the rapid turbidity changes identified with the glycerol-induced cycle of plasmolysis and recovery, HOCl-oxidized *E. coli* underwent a slow continuous decrease in turbidity over a period of several hours as measured by absorbance changes at 540 nm. The magnitude of the change was inconsequential until sufficient HOCl had been added to reduce *E. coli* viability by 90%, but then became increasingly pronounced with further

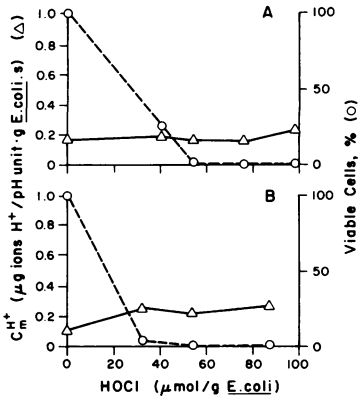


*Figure 1.* Proton conductances of HOCl-treated *E. coli*. Bacteria in 0.1 M KCl were flow-mixed with HOCl as described in the text. Proton conductances were measured at 23°C after quenching the suspension with excess $Na_2S_2O_3$ and adding reagents to give the following conditions: 50 mM KSCN, 25 $\mu$M valinomycin, 2 mM 2-deoxyglucose, 10 mg/ml carbonic anhydrase, pH 7.0. (*A*) 2.3 × $10^9$ *E. coli* 25922/ml; (*B*) 2.0 × $10^9$ *E. coli* ML-35/ml.

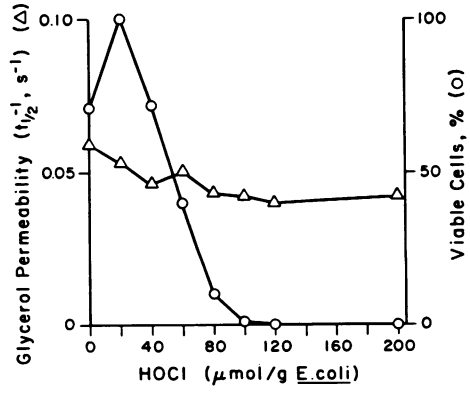


*Figure 2.* Glycerol permeabilities of HOCl-treated *E. coli* strain ML-35. 6 × $10^8$ cells/ml in 25 mM sodium phosphate, pH 7.4, 0.1 M NaCl, reacted with HOCl as in Fig. 1. After quenching, aqueous glycerol was added to give final concentrations of 0.8 M glycerol, 8.3 × $10^8$ bacteria per ml, and the reaction was followed at 23°C.

HOCl addition. Comparable results were obtained for glucose-grown and glycerol-grown cells.

*$^{14}$C-Radiolabeled uptake in E. coli 25922.* HOCl and $NH_2Cl$-induced loss of $^{14}$C-radiolabeled substrate accumulation was measured for the $H^+$-proline, $H^+$-TMG, glutamine, and leucine-isoleucine-valine transport systems. Typical results on single growth cultures are given in Figs. 3 and 4. Although for each experimental point concurrent pour-plate viability and substrate uptake was measured, to improve clarity uptake of individual substrates is compared in the figures to relative viability averaged over all the runs at a given dose level of oxidant. Comparable results were obtained with other growth cultures; each series was repeated three to four times.

For both oxidants, loss of metabolite transport capabilities coincided approximately with loss in cell viability. Some quantitative differences are apparent, however. With HOCl, the titrimetric curves for transport loss slightly precede viability curves, while with $NH_2Cl$, transport loss generally follows viability. Hypochlorous acid is capable of essentially complete inhibition of transport in these systems, whereas $NH_2Cl$, except for leucine uptake, was unable to completely inhibit transport, even when added in concentration levels several-fold in excess of those shown in Figs. 3 and 4. Residual transport levels for substrates measured at the point of near-zero viability in $NH_2Cl$-oxidized cells were, for proline, ~35%; for TMG, ~70%; and for glutamine, ~35%. Despite its less efficient inhibition of transport, $NH_2Cl$ is slightly more effective than equimolar HOCl at reducing viability under these conditions. Similar conclusions were previously reached from the observation that exogenously added amines potentiate chloride-dependent MPO-mediated killing of *E. coli* (29).

*o-Nitrophenylgalactoside hydrolysis.* Both *E. coli* ML-35 and 25922 exhibit accelerated rates of hydrolysis of externally added ONPG when treated with HOCl. Typical results for single growth cultures are given in Fig. 5. These graphs show both whole cell and lysed cell ONPG hydrolysis rates plotted with cellular viability against HOCl. Lysed cell ONPG hydrolysis rates measure total β-galactosidase activity in the cells; they exceed whole cell ONPG hydrolysis rates, for which ONPG entry into the cell is rate-limiting (30). β-Galactosidase activity remains largely undiminished by HOCl sufficient to kill >95% of the cells, but continued addition of HOCl beyond this point causes progressive loss of activity. The enzyme is highly susceptible to inactivation by HOCl. For example, β-galactosidase in 25 mM phosphate buffer, pH 7.4, containing 0.1 M NaCl, at a concentration level comparable to the lysed cell preparations is completely inactivated upon addition of 5 μM HOCl.

Neither strain of *E. coli* was protected from HOCl-induced

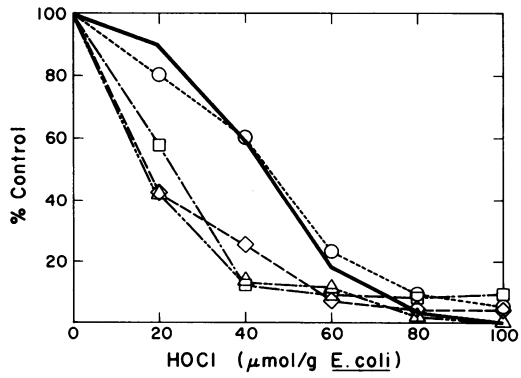


*Figure 3.* $^{14}$C-labeled metabolite uptake by HOCl-treated *E. coli* 25922. Reaction conditions are given in the text. Solid line, relative cell viability; circles, [$^{14}$C]TMG uptake, bacteria at $8 \times 10^8$ cells/ml, 100% control is $2.3 \times 10^4$ cpm; squares, [$^{14}$C]leucine uptake, bacteria at $6 \times 10^8$ cells/ml, 100% control is $2.1 \times 10^4$ cpm; diamonds, [$^{14}$C]glutamine uptake, $9 \times 10^8$ cells/ml, 100% control is $2.2 \times 10^4$ cpm; triangles, [$^{14}$C]proline uptake, $6 \times 10^8$ cells/ml, 100% control is $2.4 \times 10^4$ cpm.

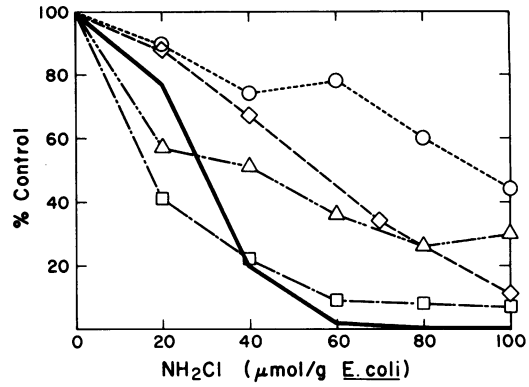


*Figure 4.* $^{14}$C-labeled metabolite uptake by $NH_2Cl$-treated *E. coli* 25922. Reaction conditions are given in the text; bacteria were quenched with $Na_2S_2O_3$ after 5 min of exposure to $NH_2Cl$. Conditions and symbols are the same as in Fig. 3, except 100% control values were: for [$^{14}$C]TMG, $1.6 \times 10^4$ cpm; for [$^{14}$C]leucine, $2.2 \times 10^4$ cpm; for [$^{14}$C]glutamine, $2.0 \times 10^4$ cpm; for [$^{14}$C]proline, $1.8 \times 10^4$ cpm.

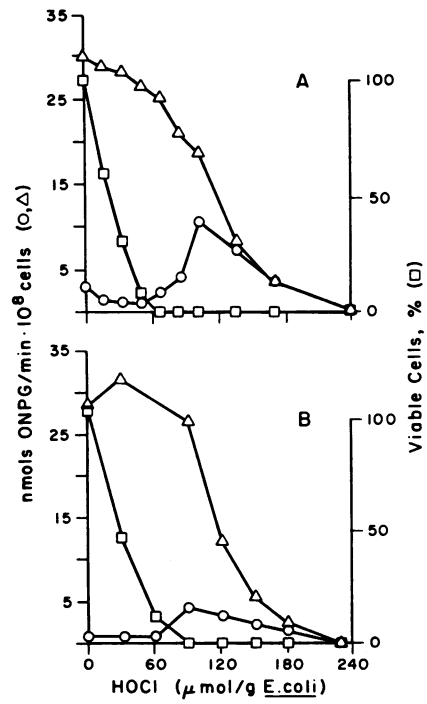


*Figure 5.* ONPG hydrolysis in HOCl-treated and lysed *E. coli*. (*A*) $6 \times 10^8$ *E. coli* 25922/ml; (*B*) $7 \times 10^8$ *E. coli* ML-35/ml. Reaction with HOCl in 25 mM sodium phosphate, pH 7.4, 23°C. Enzymatic assay as described in the text. Circles, experimentally determined rates for intact cells; triangles, rates for cell homogenates.

ONPG hydrolysis by addition of the lactose permease inhibitors thiodigalactoside (TDG) or melibiose. The inhibitor concentration levels used, 2 mM TDG and 10 mM melibiose, are sufficient to block ONPG uptake by the lac permease in untreated *E. coli* (31). Incubation of cells with 1 mM *N*-ethylmaleimide (NEM) for 30 min before HOCl oxidation was also ineffective in altering the dynamics of cellular ONPG hydrolysis; NEM is thought to bind a critical sulfhydryl group in the permease, blocking transport (32).

The time dependence of HOCl-induced ONPG hydrolysis was studied in *E. coli* ML-35. Only a slight increase in ONPG hydrolysis over control values was observed when measured immediately after exposure of cells to sufficient HOCl to just abolish viability, but subsequent measurement showed progressive increase in hydrolysis rates over periods exceeding 1 h (Fig. 6). Reproducibility of maximal ONPG hydrolysis rates induced by HOCl in both *E. coli* strains was very poor, despite careful control of growth conditions, preparation, and manipulation of HOCl-oxidized cells. *E. coli* ML-35 gave maximal rates that varied from 5 to 20 nmol ONPG/min per $10^8$ cells, but *E. coli* 25922 gave no increase in hydrolysis rate over control levels in approximately half of the 20 sets of experiments performed with this strain. ~25–40% of the total ONPG hydrolase activity from *E. coli* ML-35 was found in the supernatant fraction when cell suspensions were centrifuged (Fig. 6), which indicates that much of the β-galactosidase giving rise to reaction was extracellularly localized.

Treatment of cells with $NH_2Cl$ did not give rise to detectable changes in ONPG hydrolysis rates on the timescale investigated. Fig. 7 demonstrates the effect of $NH_2Cl$ and HOCl on ONPG hydrolysis in the same growth culture of *E. coli* ML-35. Also shown is the protective effect of thiosulfate ion upon $NH_2Cl$-induced cellular inactivation. If bacteria exposed to $NH_2Cl$ were immediately treated with $S_2O_3^{2-}$ ion, no loss in viability occurred (Fig. 7 *A*), but if $S_2O_3^{2-}$ addition was delayed 5 min, then the viability loss exceeded that caused by an equivalent amount of HOCl (Fig. 7 *B*).

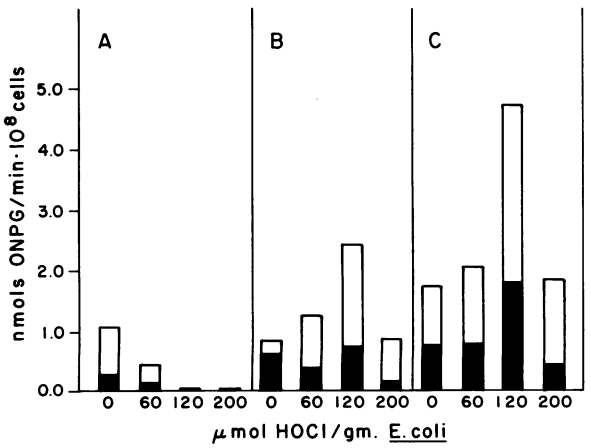


*Figure 6.* Time dependence of ONPG hydrolysis by HOCl-treated *E. coli* ML-35. HOCl and *E. coli* at $4 \times 10^8$ cells/ml were flow-mixed and reaction was immediately quenched by addition of excess $Na_2S_2O_3$. Enzymatic assays were made on whole cells suspensions (total bar height) and supernatant fractions (shaded portion of the bar) at 3 min after mixing reactants (*A*), at 30 min (*B*), and at 60 min (*C*) after reaction. Experimental details are given in the text.

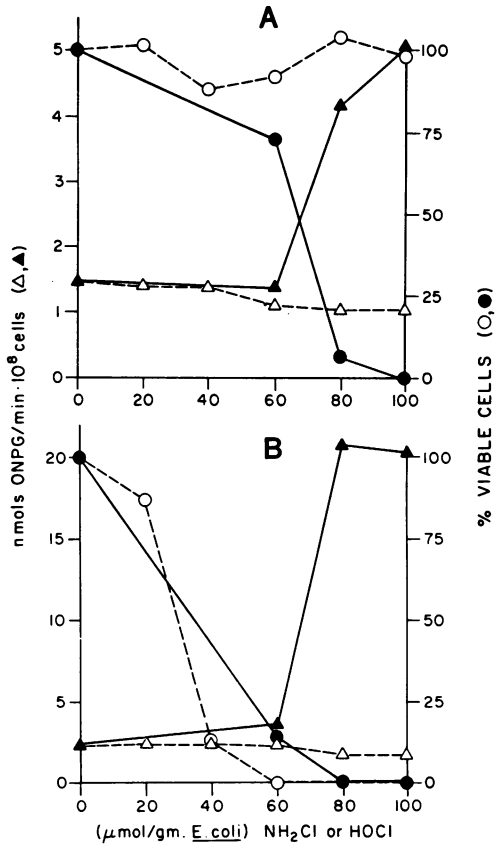


*Figure 7.* ONPG hydrolysis by HOCl and $NH_2Cl$ treated *E. coli* ML-35. Reaction conditions same as Fig. 5. (*A*) Immediately quenched with $Na_2S_2O_3$; (*B*) quenched at 5 min with $Na_2S_2O_3$. Open symbols are data points for oxidation with $NH_2Cl$; closed symbols are data points for oxidation with HOCl.

## Discussion

*Membrane permeabilities.* It is generally accepted that the cytoplasmic membrane limits the rate of cellular transport of both glycerol and protons (24, 33). In *E. coli* grown on glucose, very few pores that allow facilitated diffusion of glycerol (28) exist in the membrane so that entrance of glycerol occurs primarily by passive diffusion. Protons can enter bacteria by a variety of mechanisms which comprise both passive diffusion and systems for which their movement is coupled to metabolite and ion transport, ATP synthesis, or motility (34). Although the relative proportions of $H^+$ ion which pass the membrane by these pathways have not been determined, it is clear that the proton conductance of the membrane is, at the most, only slightly modified[2] by HOCl treatment (Fig. 1). Similarly, glycerol diffusion rates actually appear to decrease slightly with progressive oxidation of the cells (Fig. 2). In contrast, addition of lipophilic proton

2. It might be argued that a several-fold increase in $C_m^{H+}$ occurs, indistinguishable from experimental uncertainty, which nonetheless is detrimental to the cell. The proton-motive force (Δp) provides an independent measure of the membrane's capacity to act as a barrier to ion movement. No decrease in Δp is observed until HOCl in two- to threefold excess of that required to prevent colonial growth is added (Barrette, W. C., unpublished observations) indicating that at bactericidal concentrations the membrane retains its essential charge-storing function.

carriers or induction of pore-forming proteins by growth on glycerol causes marked enhancement of their rates of intercellular movement. This latter condition can be thought to mimic the consequences of introduction of lesions in the cytoplasmic membrane which might occur, e.g., if HOCl attacked structural elements. From the absence of this effect in HOCl-inactivated cells, we infer that the cytoplasmic membranes of both *E. coli* M-35 and ATC 25922 retain their generalized impermeability toward small molecules and ions. Consequently, altered rates of metabolite entry or exit (Fig. 3) must involve HOCl-induced changes in specific membrane proteins and/or loss of driving forces for their accumulation.

Our studies on HOCl-enhanced ONPG hydrolysis by *E. coli* confirm an earlier report (14) of the phenomenon for ML-35 and extend the observation to a wild-type organism (Fig. 5). Several lines of evidence suggest that the reaction is not the consequence of a generalized increase in membrane permeability, as originally proposed, but represents damage or lysis of the membranes of a subfraction of the oxidized cells. First, as discussed above, the inner membranes of the majority of the cells retain their impermeability to other small molecules and ions. In these experiments, lysis of a small percentage of the cells would imperceptibly affect the measured response of the whole population. Second, roughly 25–40% of the HOCl-induced β-galactosidase activity is extracellular under all experimental conditions (Fig. 6). The ONPG hydrolytic activity observed in whole cell suspensions always composed <10% of the ultrasonically lysed, i.e., total cytoplasmic, activity of the ML-35 strain and <30% of the ATCC 25922 strain, in instances where it was observed (the data in Fig. 5 *A* are an extreme example). Thus, only a small subset of the total cell population need be oxidatively disrupted to account for the extracellular activity. Third, the slow decrease in turbidity observed with HOCl-killed bacteria occurs within the same time domain as appearance of ONPG hydrolytic activity (Fig. 6). The optical changes may reflect cell lysis or structural changes that lead to lysis of some of the cells. An alternative possibility that HOCl has modified an otherwise inactive lactose permease in ML-35, rendering it functional, is excluded because ONPG hydrolysis is not inhibited by adding compounds that block transport by the lac porter.

*Microbicidal mechanisms.* Intracellular concentrations of active β-galactosidase, as well as those of other HOCl-sensitive (13) cytoplasmically localized biomolecules, e.g., aldolase (12), nucleotides (Barrette, W. C., Jr., unpublished observations), are unaffected by addition of bactericidal amounts of HOCl. The data on β-galactosidase presented here are titrimetric (Fig. 5), clearly identifying the relationship between loss of viability and enzyme activity. From these observations it appears that the bactericidal reactions occur on the cell envelope and not in the cytoplasm.[3]

In *E. coli* 25922, uptake of all radiolabeled substrates, whether driven by proton cotransport or ATP hydrolysis, declined sharply upon exposure to HOCl. The decrement of substrate uptake slightly precedes titrimetrically the loss of viability in all four transport systems (Fig. 3). This suggests that the microbicidal action of HOCl may involve, at least in part, the inability to acquire essential nutrients from the environment. Loss of ability to accumulate $^{14}$C-lysine by *Lactobacillus acidophilus* in response to MPO-catalyzed iodide ion peroxidation has been reported (35), which suggests that lethal iodination reactions may also involve microbial transport systems. The quantitative relationship between cellular death and transport loss was not examined in these studies, however.

Mechanisms of transport inhibition could involve direct oxidative attack of the transport proteins and/or dissipation of electrochemical gradients and cellular energy that compose the driving forces for active substrate uptake. We have recently observed that loss of cell viability in HOCl-oxidized cells also coincides with massive phosphoanhydride bond hydrolysis (Barrette, W. C., Jr., unpublished observations), which is consistent with either inhibition mechanism. However, titrimetric loss of transport for systems requiring cotransport of protons precedes loss of the proton-motive force (Δp), which suggests that for these systems, inactivation probably involves direct attack of the porters.

Other researchers have shown that killing of *E. coli* by intact or disrupted neutrophils can occur without apparent inhibition of transport of metabolites essential for macromolecular synthesis (36–38). In this instance, the mechanism is nonoxidative and thought to be mediated by a specific cationic protein whose locus of binding is the bacterial outer membrane (39, 40). Although these observations have called to question the relevance of HOCl oxidations to phagosomal toxicities (41), the media used in these studies contain relatively high concentrations of hydrophilic amino and other compounds which protect bacteria from oxidative damage by scavenging added HOCl (29). The conditions of the experiments, therefore, select for nonoxidative cidal mechanisms, rendering uncertain attempts at extrapolation to the phagosomal milieu.

*Reactivity differences between oxidants.* As a consequence of possessing a less electrophilic chlorine atom (42), $NH_2Cl$ is both less reactive and more selective an oxidant than HOCl. This differing reactivity can manifest itself in differing cellular responses. In erythrocytes, $NH_2Cl$ causes oxidation of hemoglobin to its met form, but HOCl causes primarily cell lysis (2). In *E. coli,* inactivation by $NH_2Cl$ apparently occurs without complete inhibition of substrate transport[4] (Fig. 4). The differential response of *E. coli* to $NH_2Cl$ and HOCl might be used to identify and distinguish between these agents as mediators of neutrophil bacterial toxicity. Direct oxidative attack by phagosomal-generated HOCl is suggested from the observations that both HOCl and the phagosomal reactions of *E. coli* ML-35 give rise to enhanced levels of ONPG hydrolysis, but $NH_2Cl$ does not, and

3. An ambiguity is introduced because viability is not ascertained until at least 24 h after exposure to the oxidants. Slow reactions not evident when the bacteria are examined immediately after oxidation could conceivably contribute to killing in the intervening period. A pertinent example is the observed increase in ONPG hydrolysis discussed above (Fig. 6). Nonetheless, viability provides a useful benchmark upon which to standardize the response of individual cell cultures for various oxidant doses, and the immediately detectable correlating metabolic dysfunctions that are observed with HOCl oxidation are sufficient to ensure cellular death.

4. Absence of complete transport inhibition does not necessarily imply a unique cidal mechanism for $NH_2Cl$ if some of the oxidizing equivalents can be sequestered in a form, e.g., endogenous chloramines, that escapes quenching by $S_2O_3^{2-}$ ion. Under these circumstances, it might simply be that the reactions are incomplete within the time frame of the transport measurements.[3] Oxidation by HOCl vs. $NH_2Cl$ can still be distinguished, however, on the basis of the dynamics of transport inhibition.

that bacteria appear to be killed rapidly within the phagosome[3] (43, 44), consistent with HOCl oxidation rates (21), but possibly not with the slower bactericidal reactions of $NH_2Cl$ (Fig. 7). Before such conclusions could possibly be drawn, however, the cidal reactions of all putative oxidative and nonoxidative (41) phagosomal toxins must be understood in greater detail.

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