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General Mechanism for the Bacterial Toxicity of Hypochlorous Acid: Abolition of ATP Production†

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ABSTRACT: The adenylate energy charges (EC) of *Escherichia coli* 25922, *Pseudomonas aeruginosa* 27853, and *Streptococcus lactis* 7962 rapidly fell in nutrient-rich media from values in excess of 0.9 to below 0.1 when the organisms were exposed to lethal levels of HOCl. The same cells maintained in energy-depleted states were incapable of attaining normal EC values necessary for biosynthesis and growth when challenged with nutrient energy sources after HOCl exposure. These changes correlated quantitatively with loss of replicative capabilities. Initial rates of transport of glucose, succinate, and various amino acids that act as respiratory substrates and the ATP hydrolase activity of the F_1 complex from the ATP synthase of *E. coli* 25922 also declined in parallel with or preceded loss of viability. These results establish that cellular death is accompanied by complete disruption of bacterial ATP production by both oxidative and fermentative pathways as a consequence of inhibition of inner membrane bound systems responsible for these processes.

The potent microbicide hypochlorous acid (HOCl) is generated in activated neutrophils by myeloperoxidase (MPO)[1]-catalyzed peroxidation of chloride ion (Klebanoff, 1988; Hurst & Barrette, 1989). The role of HOCl in the bactericidal action of the blood leukocytes has been extensively discussed in relation to other putative toxins (Klebanoff, 1988; Hurst & Barrette, 1989; Elsbach & Weiss, 1983; Spitznagel, 1984; Ganz et al., 1985). Molecular mechanisms of killing by these various toxins have not yet been elucidated, so it has not been possible to assign a primary role to any one of them. Nonetheless, HOCl is toxic to virtually all cell types (Klebanoff & Clark, 1978), its detection in normal stimulated neutrophils

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[1] Abbreviations: AMG, methyl α-glucopyranoside; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DTT, dithiothreitol; EC, energy charge; mRNA, messenger RNA; MPO, myeloperoxidase; PBS, phosphate-buffered saline; PMF, proton-motive force.

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coincides with degranulation, and loss of bacterial viabilities, as well as the metabolic dysfunctions described herein, occur within 100 ms after exposure to lethal levels of HOCl (Albrich & Hurst, 1982). These observations suggest that HOCl plays an important role in leukocytic disinfection.

Sites of HOCl-promoted bacterial inactivation are probably within the cellular envelope since HOCl-sensitive biomolecules located in the cytosol are not attacked until the bacteria are exposed to concentration levels severalfold higher than are lethal (Barrette et al., 1987; Albrich et al., 1981; Camper & McFeters, 1979). Inactivation does not entail disruption of the physical integrity of the plasma membrane since its intrinsic impermeability to small molecules and ions is also retained (Barrette et al., 1987; Albrich et al., 1986). However, oxidation of bacterial iron–sulfur centers (Rosen & Klebanoff, 1985) and loss of membrane-bound succinate dehydrogenase activity (Rosen et al., 1987), respiration (Albrich & Hurst, 1982; Albrich et al., 1981; Rosen et al., 1987), and transport of lactose and several amino acids (Albrich et al., 1986) all accompany inactivation by HOCl and/or the cell-free MPO–H_2O_2–Cl^- system. These results establish that membrane-localized biomolecules are vulnerable to inactivation by toxic concentration levels of HOCl. Extensive hydrolysis of cytosolic nucleotide phosphoanhydride bonds has also been found to parallel HOCl inactivation of *Escherichia coli* (Barrette et al., 1987), with the consequence that the adenylate energy charge (EC), defined as EC = ([ATP] + $^1/_2$[ADP])/([ATP] + [ADP] + [AMP]), irreversibly declines to very low levels. Because the intracellular adenylate pool levels were maintained at normal levels and net ATP hydrolysis continued well after completion of oxidative attack by HOCl, it was concluded that the mechanism is indirect, probably involving damage to the energy-transducing and/or transport proteins in the bacterial membrane.

Although prior studies (Albrich et al., 1986) have shown that transport of leucine, proline, and glutamine is inhibited in HOCl-inactivated *E. coli* 25922, these amino acids are not respiratory substrates for this strain (W. C. Barrette, Jr., unpublished observations). The relationship between transport and ATP losses is therefore not established by these studies. We report herein that HOCl abolishes ATP production in *E. coli* by inhibiting transport of the fermentative substrate, glucose, and respiratory substrate, succinate, and simultaneously inactivating the membrane-localized proton-translocating ATP synthase, the central member of the respiratory energy transduction system (Downie et al., 1979). Comparable effects are observed for the obligate aerobe, *Pseudomonas aeruginosa*, and the strict fermentor, *Streptococcus lactis*, which lead to complete loss of the capacity for ATP production by these cells.

Experimental Procedures

Reagents. Radiolabeled [U-^{14}C]-D-glucose, [2,3-^{14}C]-succinate (sodium salt), [U-^{14}C]methyl α-glucopyranoside (AMG), various U-^{14}C-labeled amino acids, and the scintillation fluor Aquasol II were purchased from New England Nuclear Corp. Nutrient broth, trypticase soy broth, tryptic soy agar, casamino acids, and agar were from Difco Laboratories. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was from Aldrich Chemical Co. Electrophoresis reagents were from Bio-Rad Co. Bovine pancreas DNase type IV, Trizma base, sodium succinate, glycerol, dithiothreitol (DTT), ATP, and the various nonradiolabeled amino acids used were obtained from Sigma Chemical Co. Hypochlorous acid was prepared by vacuum distillation of neutralized commercial bleach solutions (Albrich & Hurst, 1982) and standardized by UV spectroscopy using $\epsilon_{235} = 100$. All other chemicals were of reagent grade and used without further purification.

Bacterial Strains. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were obtained from Difco as part of their MIC set; *S. lactis* ATCC 7962 was from ATCC. Methods for storage, growth, harvest, cell density adjustment, and cell viability determinations for all the organisms were as described earlier (Barrette et al., 1988) unless otherwise indicated. Final cell concentrations were $(3 \pm 0.5) \times 10^9$ cells/mL and corresponded to A_{540}/cm of 6.0 for *E. coli* 25922 and *P. aeruginosa* 27853 and 3.0 for *S. lactis* 7962. Oxidative reactions were initiated by flow-mixing cell suspensions with HOCl, as previously described (Albrich & Hurst, 1982). Sodium thiosulfate was added no later than 15 min after mixing to stop any residual chlorination reactions.

Energy Charge Measurements. The following procedure was used to measure chemically induced loss of EC in growing bacteria: Resting cells, stored on ice in 0.1 M phosphate-buffered saline (PBS), were prewarmed at 23 °C for 5 min in the presence of an energy source (22 mM glucose or succinate) before the EC measurements were begun. After the initial EC value was determined, the cells were exposed to various concentration levels of HOCl or to 50 μM CCCP, and the time course of EC reduction was determined by periodic extraction and HPLC analysis of adenine nucleotides as previously described (Barrette et al., 1988). The procedure was modified as follows to measure the metabolic response of chemically altered resting cells when challenged with a nutrient source: Cells in PBS were prewarmed in the absence of an energy source and, after exposure to HOCl or CCCP, were incubated for 30 min before addition of the energy source to allow sufficient time for the EC to decay to its new steady-state level. Subsequent response to 22 mM added glucose or succinate was measured chromatographically as above.

Metabolite Transport. Accumulation of radiolabeled substrate was measured in parallel with EC on the same bacterial suspensions. Uptake rates were determined by filtering aliquots of the bacterial suspensions at timed intervals and counting activity retained on the filter (Barrette et al., 1988). Results are reported as percent initial uptake rate relative to control rates. Phosphorylated AMG was isolated by anion-exchange chromatography and analyzed as described in the literature (Beneski et al., 1982).

Preparation of Solubilized F_1-ATPase from E. coli 25922. A soluble cell fraction containing F_1-ATPase was prepared by differential centrifugation of broken cells according to procedures described in the literature (Futai et al., 1974). *E. coli* 25922 grown in 1 L of nutrient broth supplemented with succinate was washed and resuspended in PBS containing 10 mM $MgCl_2$. The suspension was divided into aliquots and exposed to varying levels of HOCl. Sodium thiosulfate and DNase (10 μg/mL) were added, and the cells were ruptured by using an Aminco french press with the pressure kept between 8000 and 12 000 psi. The solutions (typically 20 mL) were spun at 8000*g* in a Sorvall RC2-B centrifuge for 10 min at 4 °C to remove unbroken cells and large debris. The supernatant fraction was removed and spun at 100000*g* in a Beckman Model L5-65 ultracentrifuge for 1 h at 4 °C. The supernatant was poured off, and the remaining pellet was resuspended in 3 mL of 1 mM Tris-HCl, pH 7.5, and 0.5 mM EDTA with 10% (v/v) glycerol. The F_1-ATPase is released in its fully assembled form from the membrane in this low ionic strength buffer. This solution was incubated at 23 °C for 1 h and then spun at 100000*g* for 2 h at 4 °C. DTT and ATP (1 mM) were then added. The resulting supernatant contained F_1-ATPase; its protein concentration was typically 0.2 ± 0.1

mg/mL with an ATPase specific activity (defined below) of 25 ± 5 units/mg. Protein was determined by the Bio-Rad Bradford method because DTT did not interfere with this analysis. For solutions not containing DTT, Bradford and Lowry (Lowry et al., 1951) assays gave values that differed by less than 10%.

Measurement of F_1-ATPase Activity. ATPase activity was measured by following P_i formation as a function of time at 37 °C. The rates were linear up to times required for hydrolysis of 10% of the ATP and with protein concentration over the accessible range (see below). The assay mixtures (Futai et al., 1974) consisted of 20 mM Tris-HCl, pH 8.0, 4 mM ATP, 2 mM $MgCl_2$, and F_1-ATPase-containing fractions (3.75 μg of protein) in a total volume of 2.0 mL. Inorganic phosphate was measured once every minute for 10–20 min, and the rate was calculated from the slope of P_i versus time and reported as percent relative specific activity of untreated fractions [25 ± 5 μmol of P_i/(min·mg of protein)].

Inorganic phosphate was determined as follows (McKee & Fyfe, 1985): 52 μL of ATPase assay solution was added to 1.25 mL of zinc–molybdate reagent [75 mM zinc acetate, 11.3 mM ammonium molybdate, pH 5.0 (HCl)] in a 1.3-mL quartz cuvette. The solutions were rapidly mixed, and the absorbance at 350 nm was measured within 5–10 s. There is a large background absorbance from the reagent at this wavelength, so the assay zero time point was used as the background reference absorbance. Immediate determination of P_i was required because ATP slowly hydrolyzes in the reagent medium ($A_{350} \simeq 0.02$/min). Nonetheless, the analyses were highly reproducible, and their accuracy was verified by comparing results from solutions containing identical P_i but varying ATP concentrations. Calibration curves were constructed by using appropriate P_i concentrations in the ATPase buffer. These curves were linear to absorbancies in excess of 0.6 with a slope of 0.169 ± 0.002 μmol of P_i/A_{350} and an intercept at the origin. The range of accessible rates was 0.0005–0.02 A_{350}/min, corresponding to 3.8–15 μg total protein under our experimental conditions.

Electrophoresis Procedures. Native polyacrylamide slab gel electrophoresis of the solubilized F_1-ATPase was performed on 5% gels (Hames, 1981). The gels were run with 20 μg of protein/lane and showed a distinct band due to F_1 when stained with Coomassie Blue. Gels were also stained for ATPase activity with a molybdenum/ascorbic acid/sulfuric acid reagent following soaking for 10 min in 0.1 M Tris-HCl (pH 8.7) containing 4 mM ATP and 2 mM $MgCl_2$ (Chen et al., 1956). F_1-ATPase purified to homogeneity (W. C. Barrette, Jr., unpublished observations) migrated in the same position as the band seen in the crude fraction, thus confirming its identity. Peak areas of Coomassie Blue stained protein were determined with a Bio-Rad Model 620 gel scanner and presented as percent of the untreated control.

Results

HOCl-Induced EC Loss. Normal bacteria maintain EC levels of 0.8–0.9 in the presence of appropriate energy sources (Knowles, 1977). The EC of cells oxidized by HOCl rapidly fell from 0.8–0.9 to low steady-state values dependent on the level of HOCl exposure. Figure 1 illustrates this effect for *E. coli* 25922 grown with either glucose (A) or succinate (B) added to the growth medium and incubated with the respective energy source prior to HOCl oxidation. The EC fell to 0.4–0.5 and 0.1–0.15 within 10 min for both sets of cells after exposure to HOCl levels sufficient to reduce viability to 50% (50 μmol of HOCl/g of *E. coli*) and less than 1% (>100 μmol of HOCl/g of *E. coli*), respectively. Most of the decay occurred within the first 2–5 min after exposure; decay rates were not strongly dependent on the HOCl exposure level. The net effect was a near-quantitative conversion of ATP to AMP. The low EC attained (0.1–0.15) by oxidized cells in nutrient-rich media is comparable to that of untreated cells incubated in the absence of an energy source (Barrette et al., 1988) (Figure 2). The EC loss induced by 50 μM CCCP is also shown in Figure 1. As reported earlier (Barrette et al., 1988), the EC does not fall appreciably when CCCP is added to cells grown and warmed with glucose (Figure 1A) but falls rapidly when CCCP is added to cells grown and incubated with succinate (Figure 1B), consistent with CCCP-induced collapse of the proton-motive force and inhibition of succinate transport (Rayman et al., 1972). The rate of CCCP-induced EC collapse seen in Figure 1B is comparable to the rate attained by cells oxidized by lethal levels of HOCl.

During the period from 20 to 40 min following exposure to HOCl, the intracellular adenine nucleotide pool level, $\sum$([ATP] + [ADP] + [AMP]), increases by a factor of 3–6 (1–2

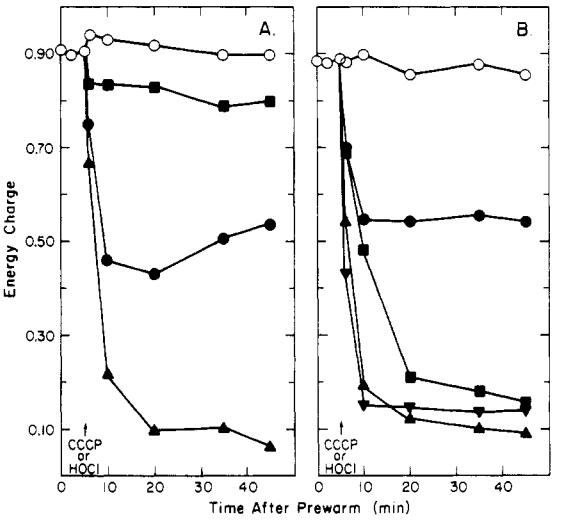

FIGURE 1: Hypochlorous acid induced energy charge decay curves for cells of *E. coli* 25922 warmed with 22 mM glucose (A) or succinate (B) prior to flow-mixing with HOCl. The cells were grown with the respective energy source added to the growth media. Symbols: 0 (○), 50 (●), 100 (▲), and 150 (▼) μmol of HOCl/g of *E. coli*; 50 μM CCCP (■). The arrows indicate the point of addition of HOCl or CCCP.

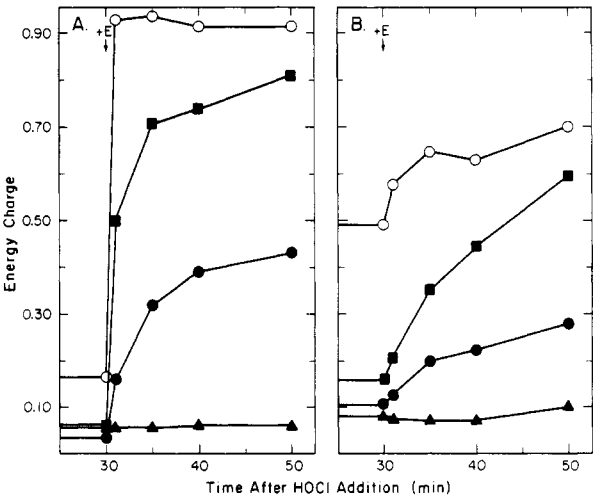

FIGURE 2: Inhibition of EC step-up by HOCl oxidation. Loss of EC step-up by *E. coli* 25922 in response to 22 mM glucose (A) or succinate (B) as a function of HOCl exposure. Symbols: 0 (○), 25 (■), 50 (●), and 100 (▲) μmol of HOCl/g of *E. coli*. Energy sources were added 30 min after HOCl, as indicated by the arrow.

to 6–8 mM) independently of the presence or absence of energy sources. The final pool level and its rate of change increase with increasing HOCl dose levels. None of the nucleotides are found in the supernatant. At higher levels of HOCl oxidation (>50 μmol of HOCl/g of *E. coli*), this pool level increase has no effect on the EC as only AMP increases substantially; however, at intermediate HOCl levels, the EC appears to rise slightly between 20 and 40 min (Figure 1A; 50 μmol of HOCl/g of *E. coli*) because both [ADP] and [AMP] increase. This pool level increase appears to be specific to HOCl exposure since the pool level remains constant during EC decreases due to short-term starvation or after addition of CCCP. Although the source of the additional nucleotides is not known, this result is what one would expect if the messenger RNA (mRNA), with a lifetime of 1–2 min (Ingraham et al., 1983), degrades as a result of HOCl-induced changes in its breakdown and/or formation rates.

HOCl-Induced Loss of EC Step-Up Capabilities. Normal bacteria increase their EC from levels as low as 0.1–0.2 to 0.8–0.9 in 1–2 min upon addition of an energy source (Barrette et al., 1988). The ability of *E. coli* 25922 to synthesize ATP after exposure to increasing concentration levels of HOCl, as measured by the EC step-up achievable when challenged with an energy source, is shown in Figure 2. Resting, i.e., nutrient-depleted, cells grown on glucose maintained an EC $\simeq$ 0.17, which fell to 0.03–0.06 in less than 25 min following exposure to HOCl (Fig. 2A). The EC in control cells immediately jumped to 0.92 upon addition of [glucose] $\geq$ 1 mM and maintained this value for at least 30 min (Barrette et al., 1988). The rates and magnitude of EC step-up decreased dramatically with exposure to increasing doses of HOCl until no step-up was observed at 100 μmol of HOCl/g of *E. coli*, an amount just sufficient to reduce viability to 1% of control levels. The EC response was independent of the time interval between HOCl and glucose additions.

Parallel behavior was observed with succinate-grown *E. coli* 25922 challenged with succinate after HOCl oxidation (Figure 2B). Some differences in the metabolic behavior of the control cells were apparent, however. The steady-state EC in resting cells was 0.5, which dropped to ~0.08 upon exposure to lethal doses of HOCl, and, unlike glucose-grown cells, the magnitude of succinate-induced step-up was less in cells maintained in nutrient-depleted than in nutrient-rich media (Barrette et al., 1988) (cf. also Figures 1B and 2B).

The relationships between steady-state EC levels achievable in nutrient-rich media and viability, measured as colony-forming units, are displayed in Figure 3. For both energy sources, decreases in EC approximately paralleled viabilities. The EC step-up induced with other energy sources such as lactose, alanine, threonine, serine, asparagine, aspartate, and casamino acids was similarly completely inhibited (data not shown), although untreated cells show EC step-up responses to these compounds similar to that with glucose. In general, lethal doses of HOCl appear to prevent *E. coli* from attaining steady-state EC levels required for growth (Knowles, 1977).

Effect of HOCl Oxidation on Nutrient Transport. The ability of HOCl-oxidized *E. coli* 25922 to transport energy sources was impaired in a dose-dependent fashion. The effect of HOCl oxidation on initial transport rates of [^{14}C]glucose, [^{14}C]AMG, [^{14}C]succinate, and [^{14}C]alanine is shown in Figure 3. In all cases, the inhibition of transport preceded loss of viability and energy charge. These results are similar to other transport studies in which inhibition of uptake of glutamine, leucine, proline, and the lactose analogue TMG either preceded or fell coincidentally with loss of viability (Barrette et al., 1987). Some quantitative differences in transport inhibition of various metabolites were observed. Although the energy charge and viability curves in Figure 3 were identical within experimental uncertainty, loss of glucose uptake (Figure 3A) occurred titrimetrically before loss of succinate uptake (Figure 3B). Additionally, at low viability, greater residual transport capability remained for succinate than for glucose.

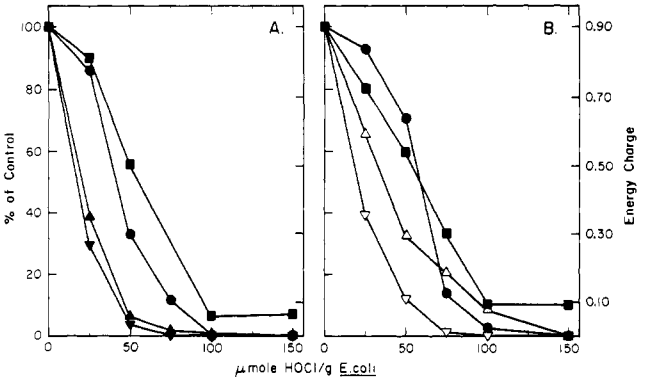

FIGURE 3: EC decay, transport inhibition, and viability loss as a function of HOCl exposure level with glucose-grown (A) or succinate-grown (B) *E. coli* 25922. Symbols: EC (■); viability (●); percent initial [^{14}C]glucose uptake rate (▲); percent initial [^{14}C]AMG uptake rate (▼); percent initial [^{14}C]succinate uptake rate (△); percent initial [^{14}C]alanine uptake rate (▽).

AMG is a nonmetabolizable glucose analogue that is specifically transported (and phosphorylated) by one (E^{III}–glu/E^{IIB}) of the two phosphoenolpyruvate-dependent glucophosphotransferases present in bacterial membranes, which is also the membrane transferase thought to be responsible for glucose transport in cells grown in glucose-containing media (Meadow et al., 1984). Collection and chromatographic analysis of the transported AMG from the cells bleached as in Figure 2 showed that all of the AMG was phosphorylated; thus, HOCl does not induce transport without phosphorylation. Loss of AMG transport indicates that at least the glucose-specific phosphotransferase was inhibited; if the other phosphotransferase (E^{IIA}/E^{IIB}) (Meadow et al., 1984) is present, it too was inhibited, since total glucose transport activity was lost in parallel with AMG transport inhibition.

Inactivation of the ATP Synthase by HOCl. To further characterize the HOCl-promoted loss of EC response to added nutrients, the F_1 complex of the proton-translocating ATP synthase, the enzyme catalyzing reversible energy-linked cellular ATP formation (Downie et al., 1979), was partially purified from oxidized *E. coli* and its ATPase activity measured. The results in Figure 4 show that HOCl inhibition of ATPase activity in succinate-grown *E. coli* 25922 coincided with loss of viability; similar results were obtained for glucose-grown *E. coli*. The ATPase activity decreased in a biphasic manner, falling sharply to 20–30% activity at lower levels of HOCl and then more slowly to zero activity at higher HOCl levels. The activity was less than 5% at an HOCl dose that reduces viability to 1% and could not be regenerated by adding excess dithiothreitol. ATPase activity was not lost to the medium as a consequence of HOCl-induced dissociation of an active F_1 complex from the membrane because the activity of the supernatant from which F_1-ATPase-containing membranes had been removed remained unchanged over the course of the oxidative titration.

Native polyacrylamide gel electrophoresis of F_1-containing fractions prepared from *E. coli* 25922 gave only one protein band staining for ATPase activity, which comigrated with the band obtained after purifying F_1 to homogeneity (Figure 5). The Coomassie Blue staining intensity of F_1 in HOCl-exposed

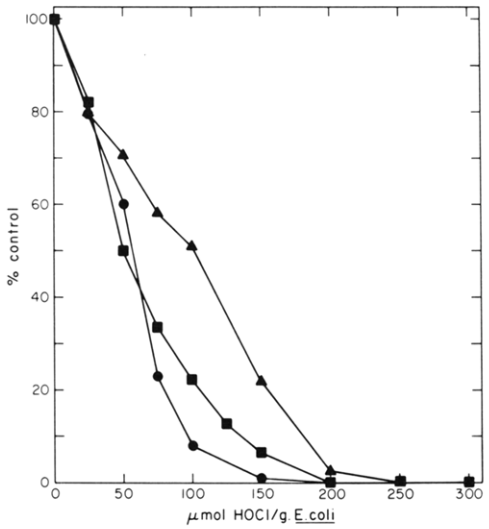

FIGURE 4: F_1-ATPase activity and composition and viability loss in HOCl-treated succinate-grown *E. coli* 25922. Symbols: viability (●) [12]; F_1-ATPase activity (■) [10]; integrated Coomassie Blue stain intensities (▲) [4]. Data are averages based upon the number of runs given in brackets; mean deviations from the average were approximately ±10%.

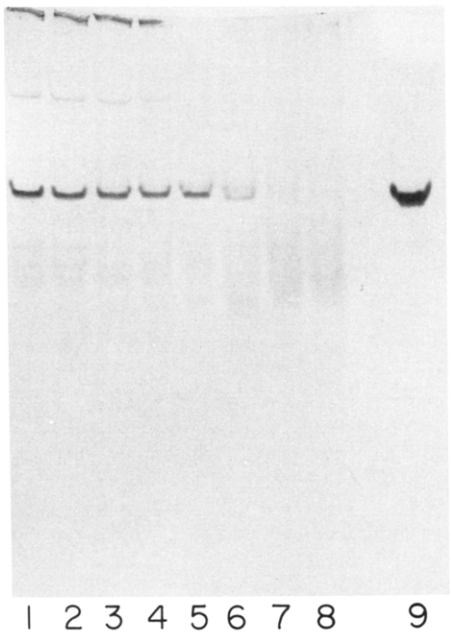

FIGURE 5: 5% native PAGE of F_1-ATPase-containing fractions after exposure to 0, 25, 50, 75, 100, 150, 200, and 250 μmol of HOCl/g of *E. coli* in lanes 1–8, respectively. Pure F_1-ATPase was run in lane 9. Protein was stained with Coomassie Blue as indicated in the text. The gel was dried between cellophane and photographed. Gels were developed for 4 h at 10-mA current.

fractions decreased upon exposure to increasing HOCl dose levels to the point that the band disappeared from fractions devoid of ATPase activity. No new bands appeared, nor did any preexisting bands increase in intensity as a consequence of HOCl treatment. Additionally, the corresponding cytosolic fractions did not show the appearance of F_1 or other new protein bands. Thus, F_1 or its high molecular weight fragments, active or inactive, are not displaced from the membrane to the cytosol. The inactivation mechanism was complex, as is apparent from the lack of congruence of titrimetric loss of ATP hydrolase activity and the F_1 complex integrity (Figure 4). Specifically, the greater sensitivity of hydrolase activity to HOCl at lower dose levels suggests that oxidative modification of the intact F_1 complex can cause partial loss of activity

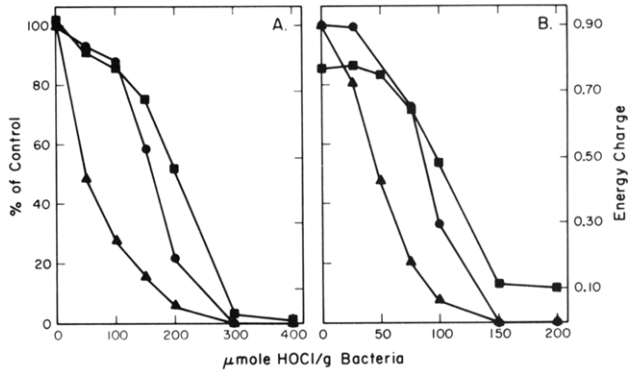

FIGURE 6: Loss of viability, nutrient transport, and maximal nutrient-promoted EC after reaction with HOCl with *S. lactis* 7962 (A) and *P. aeruginosa* 27853 (B). Symbols: viability (●); EC (■); percent initial [^{14}C]glucose (A) or [^{14}C]succinate (B) uptake rate (▲).

but that more extensive oxidation leading to subunit disaggregation is required for complete inhibition of the enzyme.

Effects of HOCl on Other Bacteria. Figure 6 displays viabilities, EC step-up, and nutrient uptake as a function of HOCl exposure for *P. aeruginosa* 27853, a Gram-negative, obligate aerobe, and *S. lactis* 7962, a Gram-positive, aerotolerant anaerobe. For both organisms, the EC step-up, viability, and energy source transport were inhibited in the same manner as with *E. coli*. Glucose or succinate did not stimulate an EC step-up in *P. aeruginosa* or *S. lactis*, respectively, as expected on the basis of their metabolic requirements. The time dependence of the EC loss measured for *P. aeruginosa* 27853 in succinate-rich media was the same as for *E. coli*. Other experiments have shown that respiration measured in *P. aeruginosa* 27853 with succinate as energy source declined in parallel with loss of viability (W. C. Barrette, Jr., unpublished observations). In addition, uptake of several amino acids was inhibited in parallel with viability loss (data not shown). These results suggest that the lesions caused by HOCl in *E. coli* 25922 occur in these organisms as well, with the consequent irreversible destruction of their energy-producing capability.

Discussion

Cellular biosynthesis appears to be under tight regulation by cytosolic nucleotides. Accordingly, when carbon nutrient sources are ample and phosphorylation levels are high, anabolic processes are favored, but these pathways shut down in favor of catabolic cellular maintenance reactions as the energy sources become limiting and phosphorylation levels decline. The EC has been proposed as a quantitative index of nucleotide regulation (Atkinson, 1968). Typically, with EC levels of 0.8–0.95, cells synthesize protein and replicate at near-maximal rates, but these functions cease when the EC declines below these values (Knowles, 1977; Chapman & Atkinson, 1977). We have shown (Figure 1) that HOCl-oxidized *E. coli* 25922 was unable to maintain its characteristically high EC in a carbon-rich environment and that the average EC fell proportionately with viability to levels comparable to energy-starved, or resting, cells. Furthermore, when several strains of resting bacteria were oxidized prior to addition of an energy source, the EC failed to rise to normal levels upon nutrient addition, but again achieved values that were proportionate to the remaining viability (Figures 2, 3, and 6). These observations support an earlier conclusion from studies on HOCl inactivation of *E. coli* 25922 that the EC value achievable in step-up experiments is the important parameter to measure when quantifying cellular metabolic competence (Barrette et al., 1988) and demonstrate more generally that HOCl-inactivated bacteria cannot respond to energy sources, but are

confined metabolically to the equivalent of their resting states. Since an EC cannot be reached that would allow net protein synthesis, repair of the damage inflicted is presumably impossible, so that the damage constitutes a fatal lesion.

The failure to achieve and maintain high steady-state EC values can arise by decreased rates of production of ATP and/or increased rates of its consumption (Chapman & Atkinson, 1977). Several lines of evidence suggest that oxidative inactivation by HOCl causes principally decreased ATP production:

First, the half-time for EC decay to new steady-state values following HOCl addition to *E. coli* 25922 is of the order of a few minutes (Figure 1A). The lethal reactions of HOCl with *E. coli* are complete within 100 ms (Albrich & Hurst, 1982), indicating that the slower rate of net ATP hydrolysis measures the metabolic response, not the primary oxidation process. This decay rate is of the same magnitude as the normal ATP pool turnover time reported for slowly growing bacteria (Chapman & Atkinson, 1977) and is also the response time observed upon addition of the protonophore CCCP to succinate-metabolizing *E. coli*. CCCP inhibits respiratory ATP synthesis by collapsing the transmembrane proton gradient driving the ATP synthase. However, it does not induce the intact ATP synthase to hydrolyze ATP in a "futile" attempt to reestablish the proton gradient since the EC levels of glucose-metabolizing bacteria are unaffected by CCCP addition (Barrette et al., 1988) (Figure 1B) as are their rates of PEP-dependent glucose transport and viabilities (Barrette et al., 1988). Because the HOCl-induced rate of EC change in respiring organisms is the same as that caused by immediate interruption of oxidative phosphorylation and because HOCl does not enhance rates of ATP production by oxidative phosphorylation in damaged cells, a conclusion based upon the absence of increased respiration (Albrich & Hurst, 1982), HOCl oxidation cannot introduce any major new pathways for ATP hydrolysis.

Second, HOCl oxidation completely inhibited the active transport of energy sources for ATP production at dose levels reducing the viabilities to 1% and the EC to 0.1. In the absence of endogenous energy stores, this transport constitutes the first step for both fermentative and respiratory pathways for ATP generation. We have now demonstrated this response for the three recognized mechanisms of metabolite transport in bacteria (Meadow et al., 1984; Rosen & Kashket, 1978), i.e., proton cotransport, ATP-driven transport, and phosphenolpyruvate-dependent group translocation. In these studies, inhibition of glucose, other sugar, and amino acid transport typically preceded titrimetric loss of viability, suggesting that nutrient transport was not limiting under the experimental conditions, i.e., that extensive damage to the transport systems was required before bacterial viabilities were affected.

Third, the ATPase component of the enzyme (F_1) that catalyzes respiration-linked phosphoanhydride bond formation between ADP and P_i was inhibited in parallel with loss of viability (Figure 4). Because this enzyme is reversible and the intracellular site of ATP hydrolase and synthase activities are identical in the intact complex (Senior & Wise, 1983), inhibition of F_1-ATPase activity undoubtedly indicates that the ATP synthase activity is similarly inhibited. Thus, even if some respiratory transport activity remained in HOCl-oxidized bacterial suspensions possessing low viability (cf. Figures 3 and 6), the respiratory substrate taken up could not be used to generate ATP.

The F_1-ATPase is a multimeric protein complex formed from five distinct subunits in the ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Senior & Wise, 1983). Gel chromatographic characterization of partially purified F_1 fractions from *E. coli* 25922 indicated that the F_1-ATP hydrolase activity was more sensitive to HOCl oxidation than was maintenance of the F_1 quaternary structure. Nonetheless, exposure to concentration levels of the oxidant sufficient to completely inhibit activity also caused dissociation of the enzyme into its component subunits (Figure 5). In this sense, the titrimetric data appear roughly biphasic, with partial inhibition of the intact F_1-ATPase complex preceding the total loss of activity that accompanies its dissociation.

Studies with hybrid F_1 complexes assembled from mutant inactive and normal active α subunits and normal β, γ, δ, and ϵ subunits have shown that all three α subunits are required for maximal activity and that if one α subunit is inactivated by exchange with a mutant α subunit, the ATPase activity of the F_1 complex as a whole drops from 100 to 25% (Rao & Senior, 1987). Other studies led to similar conclusions concerning the β subunits (Noumi et al., 1986). Inactivation of the ϵ subunit by mutation also gave a 70% reduction in the F_1-ATPase activity, the growth rate of the mutant *E. coli* was significantly reduced, and the final stationary phase cell density achieved was less than 10% of normal (Cox et al., 1987), indicating that loss of ATPase (hence, ATP synthase) activity of this magnitude significantly impaired normal cellular function. These mutations are all modifications of a single amino acid in a given subunit. More generally, mutation within the F_1 subunits caused failure to assemble a holo-F_1 complex; of 40 F_1 mutations examined, 80% did not yield isolable F_1-ATPase complexes from the organisms (Parsonage et al., 1987). These studies suggest that HOCl-induced inhibition of F_1-ATPase could occur by damage to as few as two amino acids, with reaction at one site modifying the activity of the intact complex and, at the other, causing dissociation.

A bactericidal mechanism comprising interruption of all metabolic capabilities for cellular ATP production by oxidative inactivation of the ATP synthase and membrane-localized proteins for transport of ATP-generating metabolites is capable of accounting for the broad range of microbial toxicity exhibited by HOCl. Thus, inhibition of the sugar phosphotransferases (Meadow et al., 1984) is sufficient to block ATP formation in strict fermentors such as *S. lactis*, inhibition of the ATP synthase and respiratory substrate transport similarly blocks its formation in obligate aerobes such as *P. aeruginosa*, and the superposition of both reactions effectively prevents ATP synthesis by facultative bacteria such as *E. coli* 25922. The macromolecular sites of oxidative attack have not yet been identified, but may be essential sulfhydryl groups. The F_1 complex contains nine sulfhydryl substituents (Stan-Lotter & Bragg, 1984), the glucose transport proteins are inhibited by sulfhydryl-specific reagents (Konings & Robillard, 1982), and transport by a substantial fraction of the amino acid (Berger, 1973), ion (Willsky & Malamy, 1980), and dicarboxylic acid transport proteins (Lo et al., 1972) is known to be sulfhydryl dependent. Furthermore, protein sulfhydryl substituents are highly reactive toward HOCl (Albrich et al., 1981; Albrich et al., 1986), and loss of titrable sulfhydryl groups in bacteria exposed to HOCl or MPO–H_2O_2–Cl^- has been shown to coincide with oxidative killing (Thomas, 1979a,b). Research in our laboratory is currently directed at identifying the oxidation sites within the F_1 complex, the reactions of which may be prototypic of mechanisms of inactivation of bacterial membrane-bound proteins.

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