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# Are Respiratory Enzymes the Primary Sources of Intracellular Hydrogen Peroxide?*

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**Endogenous $H_2O_2$ is believed to be a source of chronic damage in aerobic organisms. To quantify $H_2O_2$ formation, we have generated strains of *Escherichia coli* that lack intracellular scavenging enzymes. The $H_2O_2$ that is formed within these mutants diffuses out into the medium, where it can be measured. We sought to test the prevailing hypothesis that this $H_2O_2$ is primarily generated by the autoxidation of redox enzymes within the respiratory chain. The rate of $H_2O_2$ production increased when oxygen levels were raised, confirming that $H_2O_2$ is formed by an adventitious chemical process. However, mutants that lacked NADH dehydrogenase II and fumarate reductase, the most oxidizable components of the respiratory chain *in vitro*, continued to form $H_2O_2$ at normal rates. NADH dehydrogenase II did generate substantial $H_2O_2$ when it was overproduced or when quinones were absent, forcing electrons to accumulate on the enzyme. Mutants that lacked both NADH dehydrogenases respired very slowly, as expected; however, these mutants showed no diminution of $H_2O_2$ excretion, suggesting that $H_2O_2$ is primarily formed by a source outside the respiratory chain. That source has not yet been identified. In respiring cells the rate of $H_2O_2$ production was ~0.5% the rate of total oxygen consumption, with only modest changes when cells used different carbon sources.**

Superoxide ($O_2^{\cdot-}$) and hydrogen peroxide ($H_2O_2$) are partially reduced oxygen species that are substantially more reactive than is oxygen itself. Treatments that artificially generate large amounts of these species inside living cells, including the administration of hyperoxia, redox-cycling drugs, or authentic hydrogen peroxide, can disrupt metabolism and generate mutagenic or even lethal doses of DNA damage (1, 2). Even absent such forcing conditions, some $O_2^{\cdot-}$ and $H_2O_2$ are generated when molecular oxygen chemically oxidizes reduced metabolites or enzymes inside the cell. For this reason, it has been suggested that endogenous $O_2^{\cdot-}$ and $H_2O_2$ may create the cell damage that underlies important human pathologies, including those that derive from carcinogenesis and the aging process. A key question, then, is whether $O_2^{\cdot-}$ and $H_2O_2$ are produced inside cells in doses sufficient to account for these disabilities. To answer that question we need to know both the rates at which they are formed and the doses that can cause toxicity.

Evidence for the toxicity of endogenous oxidants was first obtained in *Escherichia coli*. In the mid-1980s, Carlioz and Touati created mutant strains of *E. coli* that lack cytosolic superoxide dismutase (3). These mutants exhibited defects in amino acid biosynthesis and tricarboxylic acid cycle function that led to the discovery that superoxide can inactivate dehydratases containing iron-sulfur clusters (4–8). Analogous phenotypes were subsequently discovered in superoxide dismutase-deficient yeast (9–11). Mouse mutants that lacked mitochondrial superoxide dismutase died within 10 days, an occurrence that may also have reflected deficiencies in tricarboxylic acid function (12). These phenotypes demonstrated that endogenous superoxide production is sufficiently rapid to poison a cell that lacks superoxide dismutase activity. Subsequent dosimetric analysis indicated that wild-type *E. coli* synthesizes just enough superoxide dismutase to keep its superoxide-sensitive enzymes predominantly active (13).

Similarly, mutants of *E. coli* that cannot scavenge endogenous $H_2O_2$ also exhibit severe growth defects (14). Although the underlying injuries have not yet been identified, the phenotype confirms that $H_2O_2$ is also generated inside the cells at rates that require the presence of scavenging enzymes.

How are the intracellular $O_2^{\cdot-}$ and $H_2O_2$ formed? Molecular oxygen is a triplet species, meaning that it cannot remove more than one electron at a time from organic molecules (15). This spin restriction has an important consequence: because molecular oxygen has a weak affinity for that first electron ($E_m$ = −0.16 V), there are few biomolecules that can spontaneously transfer an electron to it. This feature greatly diminishes the potential toxicity of oxygen. It also suggests that the respiratory chain is among the few plausible sites of $O_2^{\cdot-}$ and $H_2O_2$ formation, because its flavins, quinones, and metal centers are all univalent electron carriers of sufficiently low potential to react with oxygen.

Indeed, both $O_2^{\cdot-}$ and $H_2O_2$ have been detected as trace byproducts when mitochondrial or bacterial membrane vesicles respire *in vitro* (16–18). When mitochondrial complex III is inhibited with antimycin, electrons are rapidly transferred from its Qo site to oxygen (19). Superoxide is produced stoichiometrically, although it subsequently dismutates to produce $H_2O_2$ as a secondary product. This $O_2^{\cdot-}$ is evidently formed on the outer aspect of the mitochondrial membrane so that it would contribute to oxidative stress in the inner membrane space but not within the matrix (20). Mitochondrial complex I (NADH dehydrogenase) generates $H_2O_2$ *in vitro* either when downstream inhibitors favor reverse electron flow from reduced ubiquinone or when rotenone blocks turnover of the NADH-reduced enzyme (21). In both cases, the specific site of electron transfer to oxygen is unclear. Furthermore, it is difficult to know whether the same mechanisms of oxidation pertain when inhibitors are not present.

In contrast to the mitochondrial studies, *in vitro* analyses of

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TABLE I
*E. coli strains and plasmids*

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| BW6165 | *argE86*::Tn*10* | 53 |
| KM34 | As AN384 plus *malE52*::Tn*10* | Lab collection |
| SP41 | *fre*::*kan* . . . *zih-102*::Tn*10* | Lab collection |
| MG1655 | $F^-$ wild-type | Howard Steinman |
| JI301 | *nuo* . . . *zej-223*::Tn*10* | Lab collection |
| JI222 | Δ(*frdABCD)8* . . . *zjd*::Tn*10* | 33 |
| JI364 | Δ(*katG17*::Tn*10*)1 | 14 |
| JI372 | Δ*ahpCF′ kan*::*′ahpF* Δ(*katE12*::Tn*10*)1 | 14 |
| JI377 | Δ*ahpCF′ kan*::*′ahpF* Δ(*katG17*::Tn*10*)1 *katE12*::Tn*10* | 14 |
| LC100 | As JI364 plus *argE86*::Tn*10* | P1(BW6165) × JI364 |
| LC104 | As JI372 plus *argE86*::Tn*10* . . . Δ(*katG17*::Tn*10*)1 | P1(LC100) × JI372 |
| LC106 | Δ*ahpCF′ kan*::*′ahpF* Δ(*katG17*::Tn*10*)1 Δ(*katE12*::Tn*10*)1 | P1(JI372) × LC104 |
| LC109 | As JI377 plus pMW01 | This study |
| LC110 | As JI377 plus pFN3 | This study |
| LC114 | As JI377 plus pBR322 | This study |
| LC118 | As LC106 plus *fre*::*kan* . . . *zih-102*::Tn*10* | P1(SP41) × LC106 |
| LC126 | As LC106 plus Δ*frd(frdABCD)18* . . . *zjd*::Tn*10* | P1(JI222) × LC106 |
| LC128 | As JI377 plus pH3 | This study |
| LC132 | Δ*menA*::*cm* | This study |
| LC138 | As LC106 plus *nuo* . . . *zej-223*::Tn*10* | P1(JI301) × LC106 |
| LC141 | As LC106 plus pHS1–4 | This study |
| LC145 | As LC106 plus Δ*menA*::*cm* | P1(LC132) × LC106 |
| LC147 | As LC145 plus *ubiA420* . . . *malE52*::Tn*10* | P1(KM34) × LC145 |
| LC149 | As LC106 plus ubiA420 . . . *malE52*::Tn*10* | P1(KM34) × LC106 |
| LC150 | As LC147 plus pMW01 | This study |
| LC156 | As LC138 plus *ndh*::*cm* | P1(MW03) × LC138 |
| LC160 | As LC147 made $cm^s$ | This study |
| LC165 | As LC160 plus *ndh*::*cm* | P1(MW03) × LC160 |
| MW03 | *ndh*::*cm* pMW01 | Lab collection |
| MW11 | As JI377 plus *ndh*::*cm* | P1(MW03) × JI377 |
| GS022 | *araD139* Δ(*argF-lac)169* $\lambda^-$ *flhD5301 fruA25 relA1 rpsL150 rbsR22 deoC1* λRS45 $\phi$(*katG*::*lacZ*) | Gisela Storz |
| LC70 | As GS022 plus Δ*ahpCF′ kan*::*′ahpF* | 14 |
| LC133 | As GS022 plus pMW01 | This study |
| LC134 | As LC70 plus pMW01 | This study |
| LC137 | As GS022 plus pBR322 | This study |
| pMW01 | pBR322 plus $ndh^+$ insert | 30 |
| pFN3 | pJF119EH plus $fre^+$ insert | 54 |
| pH3 | pBR322 plus $frdABCD^+$ insert | 55 |
| pHS1–4 | pHC79 plus $sodB^+$ insert | 56 |

the *E. coli* respiratory chain have indicated that $O_2^-$ and $H_2O_2$ are generated primarily by the autoxidation of reduced dehydrogenases (22). NADH dehydrogenase I, the bacterial homologue of complex I, was not a major contributor, but the much simpler NADH dehydrogenase II reacted with oxygen at a substantial rate. Succinate dehydrogenase produced scanty but still detectable $O_2^-$. Its anaerobically synthesized isozyme, fumarate reductase, autoxidized rapidly when exposed to air. With each of these enzymes, the $O_2^-$ and $H_2O_2$ are formed when molecular oxygen adventitiously oxidizes a reduced, solvent-exposed flavin. Although the spin restriction dictates that $O_2^-$ must be the initial product of these electron transfer reactions, in most cases a second electron was transferred to $O_2^-$ before it diffused out of the active sites of these enzymes, thereby producing more $H_2O_2$ than $O_2^-$ as a product. The flavins lie in cytoplasmic domains of these membrane-bound proteins so that the $O_2^-$ and $H_2O_2$ they generate would be formed inside the intact cell.

These mechanisms were identified *in vitro*. Because enzyme behavior *in vitro* does not always represent what happens *in vivo*, it is important to test whether these enzymes are actually responsible for most $O_2^-$ and $H_2O_2$ formation inside cells. Small amounts of $H_2O_2$ are indeed released by intact state 4 mitochondria in which electrons are backed up on the respiratory chain due to the absence of ADP as a $F_1$-$F_0$-ATPase substrate. When ADP was provided or the membrane potential was otherwise dissipated, the amount of $H_2O_2$ fell to undetectable levels (23, 24). The fact that $H_2O_2$ production depends upon the respiratory state confirms that the respiratory chain is the likely site of state 4 mitochondrial $H_2O_2$ production but makes it difficult to estimate the amount of $H_2O_2$ that would be generated in actively respiring cells *in vivo*. Furthermore, recent calculations indicate that most $H_2O_2$ is scavenged by glutathione peroxidase before it diffuses out of the matrix (25). Thus, these efflux experiments may have detected only the $H_2O_2$ that was produced on the outer aspect of the mitochondrial membrane.

Until recently, it was not possible to measure the $H_2O_2$ formed inside living *E. coli*, as this bacterium contains an NADH peroxidase and two catalases and does not release the $H_2O_2$ that is generated in its cytosol (26). However, this problem can now be circumvented by the use of mutants that lack these enzymes (14). In the present study, the stepwise elimination of respiratory enzymes allowed us to appraise directly their contribution to the overall rate of $H_2O_2$ formation *in vivo*. Surprisingly, the results indicate that most $H_2O_2$ is formed elsewhere.

## MATERIALS AND METHODS

*Chemicals and Enzymes*—Catalase, cytochrome *c*, horseradish peroxidase (type II), 4-hydroxybenzonic acid, hydrogen peroxide (30% w/v), isopropyl-β-D-thiogalactopyranoside, deamino-NADH, NADH, NADPH, *o*-nitrophenyl-β-D-galactopyranoside, *o*-dianisidine, plumbagin, potassium cyanide, riboflavin, copper/zinc superoxide dismutase, and uracil were purchased from Sigma-Aldrich. Total protein was measured with Coomassie protein reagent (Pierce). β-Mercaptoethanol, EDTA, and dimethyl sulfoxide were purchased from Fisher, and Amplex Red was purchased from Molecular Probes. Water for the buffers was purified with a Labconco Water Pro PS system using house deionized water as a feedstock. Ampicillin, chloramphenicol, and tetracycline were used at 100, 20, and 14 μg/ml, respectively.

*Strain Construction*—The strains used in this study were derived

from *E. coli* K-12 and are listed in Table I. Mutant strains were constructed by P1 transduction (27). The tetracycline-sensitive version of JI377 (LC106) was created by transducing into JI372 a Δ(*katG17*::Tn*10*)1 allele linked to *argE86*::Tn*10*, selecting for tetracycline resistance, and then replacing the *argE* mutation by transduction of *argE*$^+$, with selection for arginine prototrophy. The resultant strain was confirmed to be hydroperoxidase I-deficient (a *katG* mutant) by enzyme assay (14). The *menA* null allele was created using the λ Red recombinase method (28). Transduction of the *menA* allele was done by chloramphenicol selection on Luria broth with glucose (LBg)[1] medium. Isolates were then screened by PCR for anaerobic growth on glycerol/fumarate (40 mM each). All *frd* and *menA* mutants were supplemented with uracil (1 mM) to bypass the requirement for function of the respiration-linked dihydroorotate dehydrogenase. *menA* mutants were all screened for hydroperoxidase I activity, because these genes can be co-transduced. The loss of the chloramphenicol marker in the *menA* deletion was achieved by using pCP20, which encodes FLP recombinase (28). The *frd* deletion was co-transduced with *zjd*::Tn*10* and screened for the inability to grow on minimal medium containing glycerol/fumarate (40 mM each). The *ubiA420* point mutation was co-transduced with *malE52*::Tn*10* and then screened for growth aerobically without 4-hydroxybenzoic acid (29). The undefined *nuo* point mutation was co-transduced with a *zej-223*::Tn*10* (30). The *ndh*, *nuo*, *frd*, and *fre* mutants were all screened using the appropriate enzyme assays that are described below. Transformation of all plasmids was done using the transformation and storage buffer protocol (31). Transductions and transformations into a hydroperoxidase-deficient (Hpx$^-$) background were done anaerobically. Phenotypic comparisons were always between isogenic strains.

*Cell Growth and Media*—LB (pH 7) contained (per liter) 10 g of bactotryptone (Difco), 5 g of yeast extract (Difco), and 10 g of NaCl (27). When glucose was added to LB, its final concentration was 0.2%. To prevent the photochemical formation of hydrogen peroxide, LB medium was shielded from light and used within 24 h of its preparation. Minimal growth medium consisted of minimal A salts (27), 1 mM $MgSO_4 \cdot 7H_2O$, 5 mg thiamine per liter, and a specified carbon source. Minimal glucose-casamino acids medium contained both glucose and casamino acids (2 g/liter each). L-amino acid supplements were used at a final concentration of 0.5 mM. Histidine was routinely added to minimal media for the growth of any derivative in the MG1655 background. This strain requires the addition of histidine to grow anaerobically (data not shown). To minimize the chemical production of hydrogen peroxide (14), the minimal media used in experiments (as noted) were prepared immediately before use and sterilized by filtration.

Aerobic cultures were routinely grown in flasks at 37 °C unless noted otherwise. Anaerobic cultures were grown in a Coy chamber (Coy Laboratory Products, Inc.) under 85% $N_2$, 10% $H_2$, and 5% $CO_2$. Optical densities of cultures were measured at 600 nm. For studies of growth on various carbon sources, cells were grown in minimal growth media containing 0.5 mM histidine, phenylalanine, tryptophan, and tyrosine (each) in order to foster better aerobic growth of the Hpx$^-$ strain without providing an additional carbon source.[2] Carbon sources tested were glucose (0.2%), casamino acids (2.0%), gluconate (0.2%), glycerol (40 mM), pyruvate (0.4%), lactate (0.4%), succinate (40 mM), and acetate (0.25%). Cells were first grown in standing overnight cultures because the resulting microaerobic conditions disfavored the outgrowth of suppressors of the Hpx$^-$ strain. To grow a standing culture overnight with glycerol, nitrate (40 mM) was added. All overnight cultures were then diluted to an OD of 0.005 into fresh aerobic media and grown aerobically with vigorous shaking to log phase (OD of 0.1–0.2). The glycerol/nitrate overnight culture was washed twice in minimal salts before dilution into fresh glycerol medium lacking nitrate. The rates of respiration and $H_2O_2$ formation were measured from the same cell cultures within 15 min of each other.

For β-galactosidase measurements, anaerobic overnights were grown in LB. Overnights were then diluted into anaerobic LB to an OD of 0.01. Cultures were grown to ~0.1 OD anaerobically and then shifted to aerobic conditions for one to two generations. β-galactosidase activity was then measured (see below).

*Measurement of Respiration Rate*—Respiration by whole cells was measured using a Rank Brothers digital model 10 oxygen sensor. Oxygen consumption was determined using 5 ml of log-phase cells in growth medium at 37 °C.

*Enzyme Assays*—Each data set presented in this study was derived from at least three independent experiments. All assays were performed on log-phase cells at an $OD_{600}$ of 0.1–0.2.

*β-Galactosidase*—Cultures were centrifuged, and pellets were washed with 50 mM cold potassium $P_i$ buffer (pH 7). Cells were resuspended in 50 mM potassium $P_i$ buffer (pH 7) at one-tenth the culture volume and lysed by sonication. Cell debris was removed from crude extracts by centrifugation at 13,000 × *g* for 20 min. β-Galactosidase activity was assayed in a 1.2-ml reaction consisting of 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml), extract, and Z buffer (27) at 28 °C. Absorbance was monitored at 420 nm.

*Flavin Reductase*—Cells were centrifuged and washed in cold 50 mM Tris-HCl buffer (pH 7.8) twice. Final resuspension was in one-hundredth of the original culture volume in cold 50 mM Tris-HCl buffer (pH 7.8). Cells were lysed by passage through a French pressure cell. Cell debris was removed by centrifugation at 13,000 × *g* for 20 min. Membranes were then removed by ultra-centrifugation (100,000 × *g* for 2 h). The soluble fraction was assayed for flavin reductase activity at room temperature by monitoring $A_{340}$ in the presence of 0.25 mM NADPH and 15 μM riboflavin (32).

*Fumarate Reductase*—Anaeobically grown log-phase cultures were incubated on ice for 10 min with 150 μg/ml chloramphenicol. This prevented the induction of succinate dehydrogenase during subsequent aerobic processing. Cells were then centrifuged at 10,000 rpm for 10 min, and pellets were washed twice with cold 50 mM potassium $P_i$ buffer (pH 7.8). Cells were lysed by passage through a French pressure cell, and cell debris was removed. Inverted membrane vesicles were then isolated from the supernatant by ultra-centrifugation at 100,000 × *g* for 2 h. The inverted vesicles were resuspended in cold 50 mM potassium $P_i$ buffer (pH 7.8) at 0.5% the original culture volume. The inverted vesicles were then assayed for succinate:plumbagin oxidoreductase activity at room temperature at $A_{550}$ in the presence of 0.4 mM plumbagin, 3.3 mM potassium cyanide (pH 7.8), 20 mM succinate, and 10 μM cytochrome *c* (33).

*NADH Dehydrogenase I*—Cells were centrifuged and washed twice with cold 50 mM MES buffer containing 10% glycerol (pH 6). Final resuspension was in one-fortieth the original culture volume. Cells were lysed by passage through a French pressure cell, and cell debris was removed. Inverted membrane vesicles were then isolated from the supernatant by ultra-centrifugation at 100,000 × *g* for 2 h. The inverted vesicles was resuspended in cold 50 mM MES buffer with 10% glycerol (pH 6) at one-fortieth the original culture volume. Vesicles were assayed immediately, because NADH dehydrogenase I activity declines when membranes are stored on ice. The inverted vesicles were assayed for NADH dehydrogenase activity at $A_{340}$ with 200 μM either deamino-NADH or NADH as the substrate. Deamino-NADH is a substrate for NdhI but not for NdhII (34). NdhI utilizes deamino-NADH and NADH with equal efficiency (30).

*NADH Dehydrogenase II*—Inverted membrane vesicles were isolated as described above. The inverted vesicles were resuspended in cold 50 mM potassium $P_i$ buffer (pH 7.8) at one-fortieth the original culture volume. Vesicles were held on ice overnight to eliminate the activity of NdhI activity, which is unstable at this pH. The inverted vesicles were then assayed for NADH dehydrogenase II activity at $A_{340}$ in the presence of 100 μM plumbagin, 3 mM potassium cyanide (pH 7.8), and 200 μM NADH (33).

*Superoxide Dismutase*—Cells were pelleted at 10,000 rpm for 10 min. Cells were then washed in cold 50 mM potassium $P_i$ buffer (pH 7.8) and resuspended in one-hundredth the original culture volume in 50 mM potassium $P_i$ buffer (pH 7.8) with 0.1 mM EDTA (pH 8). Extracts were made by passage through a French pressure cell, and debris was removed by centrifugation at 13,000 × *g* for 20 min. Superoxide dismutase was assayed using the xanthine oxidase/cytochrome *c* method (35).

*$H_2O_2$ Measurements*—Cells were grown for at least four generations (to an OD of 0.1–0.3). This preculture typically was aerated; however, when experiments included strains lacking quinone (which cannot grow aerobically), the preculture medium was anaerobic. Log-phase cells were immediately pelleted by room temperature centrifugation at 4,000 × *g* for 5 min. Because components of complex media interfere with $H_2O_2$ measurements, cells grown in LBg were washed and assayed in fresh, prewarmed minimal media containing 0.02% of both glucose and casamino acids. There is no loss of respiration due to this medium switch (data not shown). Cells grown in minimal media did not require washing and were resuspended after pelleting in the same minimal media containing one-tenth of the original concentration of carbon source. The lower concentration of carbon source was used in the assay because some carbon sources autoxidize and thereby contribute to $H_2O_2$ formation (14).

[1] The abbreviations used are: LBg, Luria Broth with glucose; Frd, fumarate reductase; Hpx$^-$, hydroperoxidase-deficient; MES, 4-morpholineethanesulfonic acid.

[2] J. Sobota and J. A. Imlay, unpublished data.

Cells were finally resuspended at an OD of ~ 0.1 with shaking at 37 °C. At selected time points the aliquots were removed, cells were removed by 1-min centrifugation in a microfuge, and $H_2O_2$ levels were determined by the Amplex Red/horseradish peroxidase method (14). Fluorescence was measured in a Shimadzu RF Mini-150 fluorometer and converted to $H_2O_2$ concentration using a curve obtained from standard samples in the same assay medium. Rates were normalized to the OD of cells at the 20-min time point. A small amount of $H_2O_2$ is generated by the dye/horseradish peroxidase detection system itself; this amount was accounted for by the standard curves. $H_2O_2$ formation rates were also corrected for any background $H_2O_2$ formed by medium alone. These backgrounds were ≤0.001 μM $H_2O_2$/min. Measurements in pyruvate medium were corrected for the ability of pyruvate to scavenge $H_2O_2$ using the equation $d[H_2O_2]/dt$ = (cellular rate of $H_2O_2$ formation) − ($k$[pyruvate][$H_2O_2$]). $k$ was determined by measuring $H_2O_2$ concentration over time after the addition of 1.5 μM $H_2O_2$ to the assay medium ($k$ = 0.350). All rates were averaged from three separate measurements and presented with standard deviation. $p$ values were calculated using Student's $t$ test.

To increase $O_2$ concentration during $H_2O_2$ measurements, pure $O_2$ was bubbled vigorously through the resuspended cells at 37 °C during the period of measurement. Under these conditions, $H_2O_2$ formation rates were also corrected for any $H_2O_2$ formation due to bubbled media alone.

## RESULTS

*Quantitation of $H_2O_2$ Formed inside Living Cells—E. coli* mutants that lack catalase and alkylhydroperoxide reductase, an NADH peroxidase, cannot scavenge $H_2O_2$ (for brevity and because the *E. coli* catalases are denoted hydroperoxidases I and II, these triple mutants are designated in this study as hydroperoxidase-deficient, or Hpx⁻). These strains excrete into the growth medium any $H_2O_2$ that is formed endogenously (14, 26). We have modified an established horseradish peroxidase/Amplex Red assay in order to quantify the rate of $H_2O_2$ formation. Exponentially growing cultures were washed and resuspended into a 37 °C medium that had been freshly prepared in order to minimize the accumulation of $H_2O_2$ from the chemical oxidation of glucose. At selected time points the cells were removed, and the accumulated $H_2O_2$ was measured. This procedure circumvents the possibility that artifacts will arise from the direct interaction of cells with the detection system, an issue that has been raised in studies of mitochondrial $H_2O_2$ excretion (36, 37).

The Hpx⁻ strain grows progressively slower when it is cultured for an extended time in aerobic medium (14), in concert with the progressive accumulation of DNA damage.[3] We were concerned that the slowed growth might affect the rates of metabolism and $H_2O_2$ production. To minimize this possibility, the Hpx⁻ mutant was grown to log phase anaerobically and then subcultured into fresh aerobic medium containing amino acids. This regime allowed the mutant to grow almost as well as the wild-type for several subsequent generations (Fig. 1*A*). At the point when cultures were harvested for measurements of $H_2O_2$ production, the respiration rate was approximately that of the wild-type strain.

Exponentially growing cultures generated ~0.5 μM $H_2O_2$/min per $OD_{600}$ in glucose medium. The vast majority of this $H_2O_2$ was formed by the bacteria, because the rate was >95% lower in the sterile medium (Fig. 1*B*). By normalizing the rate of $H_2O_2$ production to the cytoplasmic volume of the cells, we calculate a rate of 10–15 μM/s. This rate is about twice what was estimated through extrapolation of the rate at which $H_2O_2$ was formed by respiratory vesicles *in vitro* (18). Therefore, either those experiments underestimated the rates at which respiratory enzymes autoxidize, or another substantial source of $H_2O_2$ exists in *E. coli*. Several avenues of $H_2O_2$ formation have been observed during *in vitro* studies: stoichiometric

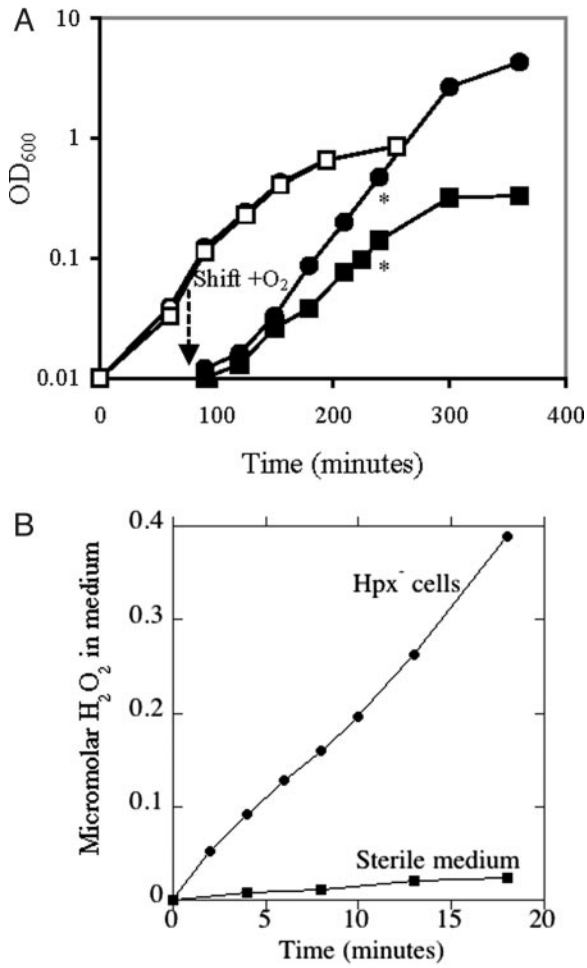


FIG. 1. **Measurement of $H_2O_2$production.** *A*, aerobic growth of an Hpx⁻ strain. Anaerobic wild-type (MG1655, shown as *circles*) and Hpx⁻ (LC106, shown as *squares*) cultures in LBg were grown into log phase (*white*). Where indicated, some cells were diluted into fresh aerobic LBg and grown aerobically in shaking flasks (*black*). Hpx⁻ strains were collected for measurement of $H_2O_2$ formation at an OD of 0.10 (*asterisks*). At this point their respiration rates approximated that of wild-type cells. *B*, $H_2O_2$ excretion by log-phase Hpx⁻ cells upon suspension in fresh glucose/amino acids medium. No $H_2O_2$ is detected in analogous cultures of Hpx⁺ cells (not shown).

$H_2O_2$ production by oxidases (38), autoxidation of excreted metabolites (39), dismutation of periplasmically generated superoxide (40), superoxide-driven chain reactions (41), and the autoxidation of redox enzymes (16–18, 22, 42). We examined each in turn to evaluate their contribution to overall $H_2O_2$ production by *E. coli*.

*$H_2O_2$ Formation Is Due to an Adventitious Reaction with $O_2$*—The rates at which flavoenzymes react *in vitro* with $O_2$ to produce $O_2^-$ and $H_2O_2$ is proportional to the concentration of dissolved oxygen (18, 22). If such adventitious reactions govern $H_2O_2$ formation *in vivo*, then increased $O_2$ levels would significantly increase $H_2O_2$ production rates. In contrast, if $H_2O_2$ were generated as a stoichiometric product of an oxidase, then increases in $O_2$ concentration would have little affect, because such enzymes are saturated by low concentrations of $O_2$.

The Hpx⁻ strain was grown to log phase aerobically. These cells were washed in fresh, prewarmed media and resuspended at an OD of ~ 0.1. $H_2O_2$ concentration was measured over time in identical cultures that were vigorously aerated with air or pure oxygen. The culture with increased $O_2$ produced ~2.5-fold more $H_2O_2$ (Fig. 2). This result suggests that the most significant source(s) of $H_2O_2$ react with oxygen adventitiously rather

[3] S. Park and J. A. Imlay, manuscript in preparation.

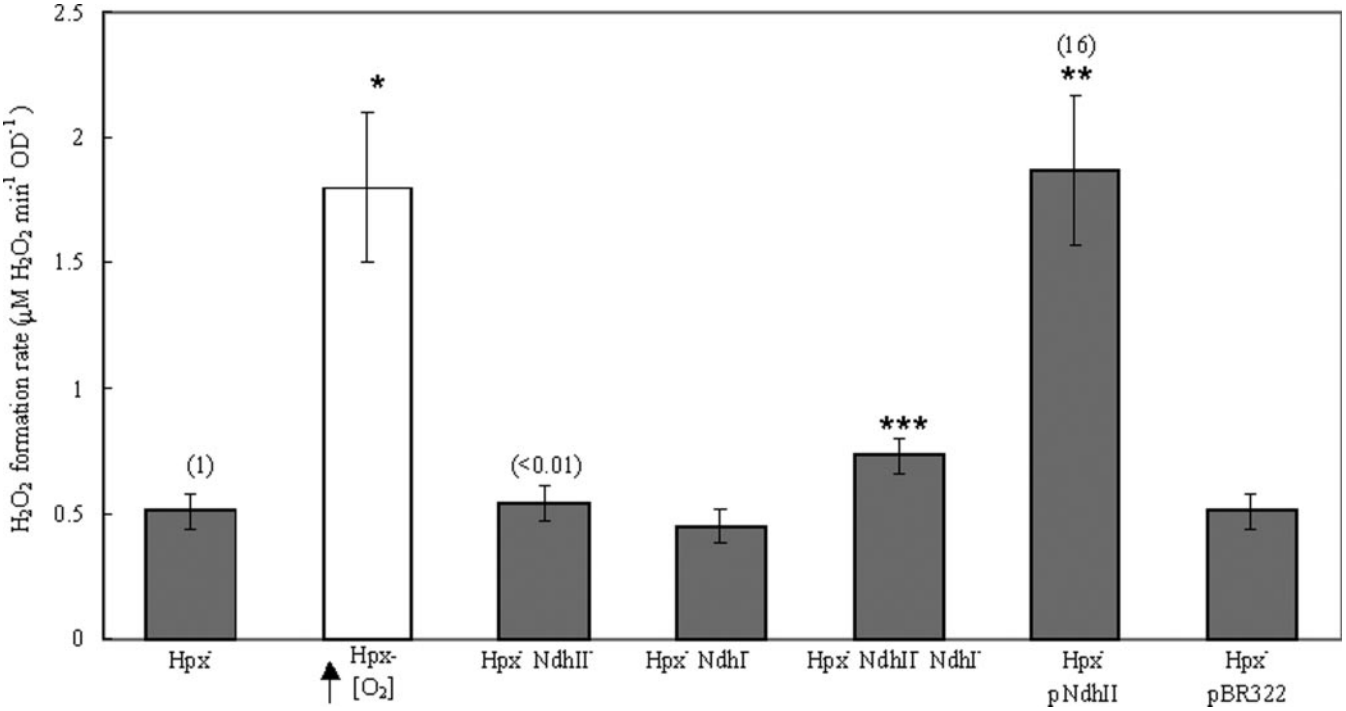


FIG. 2. **$H_2O_2$ production persists in mutants lacking NADH dehydrogenase activity.** LC106, MW11, LC138, LC156, LC109, and LC114 cultures were grown in LBg anaerobically to log phase, subcultured, and grown in aerobic LBg media to log phase. Cultures of cells with plasmids contained ampicillin throughout growth. Cells were then assayed for $H_2O_2$ formation in minimal glucose-casamino acids medium or harvested for NdhII activity. NdhII activity is normalized to that of the background strain and presented in parentheses above peroxide measurements. *, $p <$ 0.0001; **, $p < 0.004$, and ***, $p < 0.01$ *versus* background strain ($Hpx^-$).

than as an intended substrate. In fact, only one $H_2O_2$-generating oxidase is known to exist in the K12 strains of *E. coli*, the periplasmic monoamine oxidase (38), and none of its known substrates were present during these measurements.

*$H_2O_2$ Is Not Formed by an Excreted Product*—It was possible that the $Hpx^-$ strain excreted a metabolite that subsequently autoxidized to form $H_2O_2$ outside the cell. To check this possibility, cells were rapidly removed, and $H_2O_2$ formation rates were measured in the spent medium. LC106 ($Hpx^-$) was grown to log phase in aerobic minimal casamino acids glucose medium and then removed by centrifugation. The spent medium of aerobic cultures generated $<0.01$ μM $H_2O_2$/min, compared with 0.4 μM $H_2O_2$/min·OD produced by LC106 cultures (in contrast, the spent medium produced by filtration of anaerobic cultures included an unknown substance that gradually oxidized during the first 10 min when the medium was aerated).

We cannot exclude the possibility that in these control experiments some $H_2O_2$ was generated by an excreted product that autoxidized during the 60 s that was required to remove the cells. However, any metabolite that autoxidizes so efficiently could not have generated even more $H_2O_2$ when the oxygen level was raised (see above). We conclude that most or all of the $H_2O_2$ that we detected was generated inside the cells.

*Periplasmic $O_2^-$ Does Not Contribute Substantially to Endogenous $H_2O_2$*—Recent work in our lab has determined that some $O_2^-$ is formed within the periplasm during aerobic respiration.[4] Because $O_2^-$ dismutation generates $H_2O_2$, we sought to determine whether this periplasmic $O_2^-$ was responsible for much of the $H_2O_2$ that effluxes from the $Hpx^-$ cells (14). Periplasmic $O_2^-$ was measured and found to account for no more than 10% of the cellular $H_2O_2$.[4] Menaquinone is necessary for periplasmic $O_2^-$ production in these cells. For that reason $H_2O_2$ production was measured in an $Hpx^-$ strain with an additional *menA* mutation (LC132). In this mutant, $H_2O_2$ production was not appreciably decreased (Fig. 3). Therefore, the periplasmic source of $O_2^-$ did not contribute significantly to $H_2O_2$ formation.

*Superoxide-mediated Chain Reactions Are Not a $H_2O_2$ Source*—$O_2^-$ can univalently oxidize some small biomolecules, such as catechols, to form hydrogen peroxide and organic radicals, which subsequently transfer their unpaired electron to molecular oxygen. The latter reaction regenerates $O_2^-$ and propagates a chain reaction. Because $O_2^-$ dismutation ends the chain, such reactions are commonly used as assays for superoxide dismutase (43). Similarly, NADH that is bound within the active site of mammalian lactate dehydrogenase or glyceraldehyde-3-phosphate dehydrogenase can react with protonated $O_2^-$ to generate $H_2O_2$ and an $NAD^{\cdot}$ radical, which then regenerates $O_2^-$ (44, 45). These chain reactions may continue *in vitro* for many cycles, creating $H_2O_2$ at each cycle. To test the possibility that such reactions might be occurring in *E. coli*, $H_2O_2$ production was measured in concert with overproduction of iron-containing superoxide dismutase. In LC141 ($Hpx^-$ pFe-SOD), a 26-fold overproduction of superoxide dismutase did not alter the $H_2O_2$ formation rate from that of LC106 ($Hpx^-$) (data not shown). We conclude that superoxide-mediated chain reactions do not contribute significantly to $H_2O_2$ stress in *E. coli*.

*The Majority of $H_2O_2$ Formed May Not Originate from the Respiratory Chain*—The previous results led us to believe that most $H_2O_2$ is formed by the straightforward autoxidation of redox enzymes. Several flavoenzymes of *E. coli* have been identified that react with oxygen *in vitro*, forming $H_2O_2$ (18, 33, 46). To evaluate the involvement of these enzymes in $H_2O_2$ formation, we eliminated or overexpressed the structural genes of each candidate flavoenzyme in $Hpx^-$ cells.

The most compelling candidate was NADH dehydrogenase II, the primary respiratory dehydrogenase when *E. coli* grows in glucose medium. Previous *in vitro* studies found that respiring vesicles prepared from *ndh* mutants generated much less

[4] S. Korshunov and J. A. Imlay, manuscript in preparation.

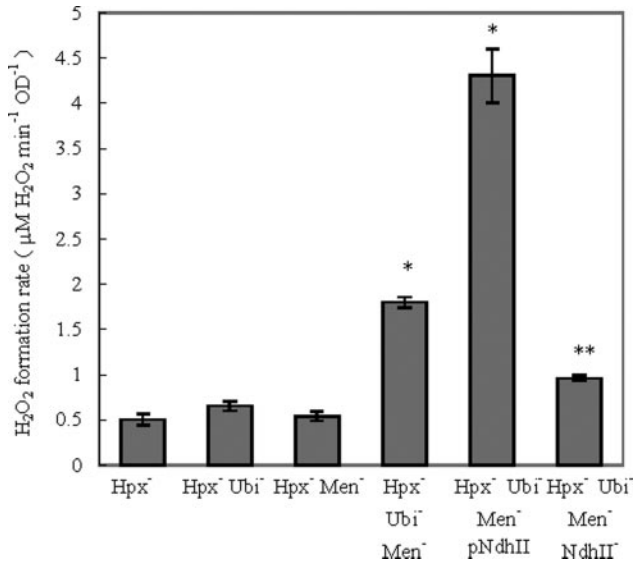


FIG. 3. **NdhII is the major source of $H_2O_2$ in cells blocked in respiration.** LC106, LC149, LC145, LC147, LC150, and LC165 cultures were grown in LBg plus uracil media anaerobically to log phase. Cultures of cells with the NdhII plasmid pNdhII (pMW01) contained ampicillin throughout growth. Cells were then assayed for $H_2O_2$ formation upon air exposure in minimal glucose (0.02%) and 20 amino acids (0.05 mM) plus uracil (0.1 mM). *, $p < 0.00005$ *versus* background ($Hpx^-$); **, $p < 0.00005$ *versus* background ($Hpx^-$ $Ubi^-$ $Men^-$).

$H_2O_2$ than did wild-type vesicles. Conversely, $H_2O_2$ production increased when NdhII was overproduced (18).

To test if NdhII was a significant source of $H_2O_2$ *in vivo*, NdhII was deleted from the $Hpx^-$ background. However, $H_2O_2$ production by this *ndh* mutant was not significantly less than that by its parent (MW11) (Fig. 2). Given the inherent error of this assay, we deduce that NdhII could contribute no more than 15% of the total $H_2O_2$ in $Hpx^-$ cells.

NdhII is responsible for ~90% of the total NADH dehydrogenase activity that is detected in membranes from *E. coli* grown in glucose medium (data not shown), with NdhI contributing the other 10%. However, inside cells the actual division of the NADH flux between NdhI and NdhII is likely to depend upon both substrate concentration and protonmotive force, and it has not been quantified. We tested whether $H_2O_2$ production could be elevated if the electron flux were forcibly directed through NdhII by eliminating NdhI. However, the addition of a *nuo* mutation (generating LC138) did not increase the rate of $H_2O_2$ formation (Fig. 2). Thus, the data indicated that another source obscures the contribution, if any, of NdhII to $H_2O_2$ production *in vivo*.

When cells are grown in glucose medium, most respiration derives from NADH oxidation by these enzymes. Thus oxygen consumption of the *nuo ndh* double mutant was <20% of that of the parent (data not shown). Surprisingly, the combination of *nuo* and *ndh* mutations did not decrease the $H_2O_2$ production rate, as would be expected if flux through the respiratory chain were needed to make endogenous $H_2O_2$ (Fig. 2). This result suggests that most $H_2O_2$ is generated outside of the respiratory chain, in contradiction to the prevailing idea in this field.

*NADH Dehydrogenase II Forms Some $H_2O_2$ in Vivo*—Because the *ndh* mutation had no discernible effect upon $H_2O_2$ production, we wondered whether the autoxidation of the enzyme that had been observed *in vitro* was entirely artifactual. Because the rate at which inverted respiratory vesicles generated $H_2O_2$ was elevated when NdhII was overproduced (18), we conducted the same experiment *in vivo*, overproducing NdhII 16-fold in the $Hpx^-$ background. Initial observations were consistent with increased $H_2O_2$ production, as the doubling time in

TABLE II
*Endogenous $H_2O_2$ from NdhII enhances OxyR activation*

| Strain[a] | β-Galactosidase activity |
|---|---|
| | *units/mg* |
| Wild-type | 0.03 ± 0.01 |
| pBR322 | 0.04 ± 0.01 |
| pNdhII[b] | 0.06 ± 0.01 |
| $Ahp^-$ | 0.57 ± 0.1 |
| $Ahp^-$ pNdhII[b] | 0.92 ± 0.1 |

[a] All strains were isogenic and harbored a λRS45 (*katG*::*lacZ*). Cells were grown in LBg. The same conditions were used for NdhII activity and $H_2O_2$ formation measurements.
[b] pNdhII = pMW01.

the aerobic medium increased from an already slow 33 min in the $Hpx^-$ strain to 44 min in the overproducer. In anaerobic media these strains grew at the same rate as the wild-type strain (data not shown). $H_2O_2$ production was then tested directly. The NdhII-overproducing strain formed ~3.5-fold more $H_2O_2$ than the parental $Hpx^-$ strain with or without the empty vector (Fig. 2).

Earlier studies showed that NdhII generated $H_2O_2$ most rapidly when membrane vesicles were prepared from mutants lacking ubiquinone (18), apparently because the absence of the downstream acceptor caused electrons to remain on the autooxidizable flavin of the enzyme. The same effect was observed *in vivo*; the quinoneless (*menA ubiA*) $Hpx^-$ mutant (LC147) exhibited a substantial increase in $H_2O_2$ production (Fig. 3). Upon the addition of an *ndh* mutation, the rate of $H_2O_2$ formation was again diminished. Conversely, overproduction of NdhII in the quinoneless $Hpx^-$ mutant (LC150) resulted in an even greater (9-fold) increase in $H_2O_2$ production. These data follow the pattern that had been observed *in vitro* and definitively show that NdhII can generate $H_2O_2$ *in vivo*.

*Physiological Evidence of Significant $H_2O_2$ Production from NdhII*—To support the conclusion that overproduced NdhII generates $H_2O_2$ in *E. coli*, we employed a *katG*::*lacZ* fusion, which serves as a reporter of OxyR activity (14). In *E. coli*, OxyR directly senses steady-state $H_2O_2$ concentration and, when it rises, positively activates transcription of a defensive regulon that includes *katG* (47). The *katG*::*lacZ* expression was elevated 10- to 15-fold in a strain lacking Ahp, the primary scavenger of endogenous $H_2O_2$ (Table II and Ref. 14). The addition of the NdhII-overproducing plasmid raised β-galactosidase activity further. No effect was observed in Ahp-proficient strains.

In sum, the experiments with NdhII confirm that the enzyme reacts with oxygen *in vivo* as it does *in vitro*. However, the results indicate that it is evidently only a minor source of endogenous $H_2O_2$.

*Fumarate Reductase Forms Little $H_2O_2$ When Anaerobic Cells Are Aerated*—The other respiratory enzyme that was identified as an $H_2O_2$ source *in vitro* was fumarate reductase (Frd). Frd is a member of the anaerobic respiratory chain and would not have been present in the preceding experiments; it is induced when oxygen is absent. Because this enzyme readily autoxidizes when it is exposed to air (33), we tested the possibility that Frd would generate substantial $H_2O_2$ when anaerobic cells are abruptly aerated. We grew the $Hpx^-$ mutant anaerobically to log phase, resuspended it in fresh glucose-fumarate medium, and then aerated the culture. The rate of $H_2O_2$ production was not substantially different from that of cultures grown continuously in aerobic medium. 8-fold overproduction of Frd increased $H_2O_2$ production only 1.8-fold (Fig. 4). This moderate acceleration of $H_2O_2$ formation presumably did not derive from an increased biosynthetic burden *per se*, because a far greater overproduction of β-lactamase had no effect upon $H_2O_2$ rates (Fig. 2).

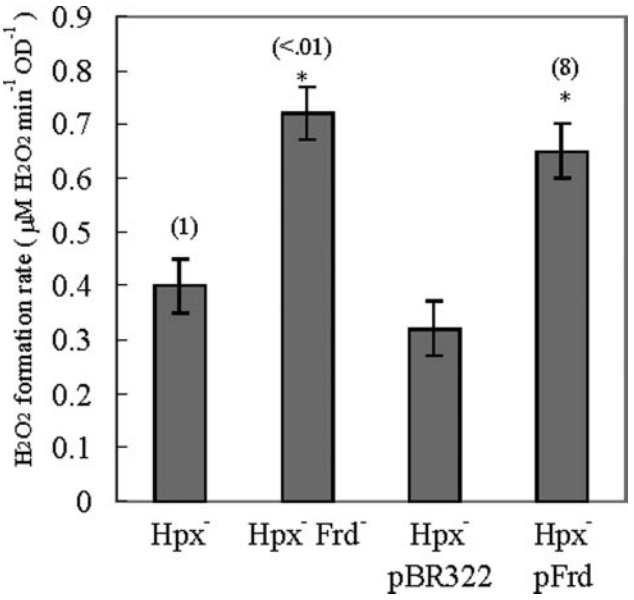


FIG. 4. **Effect of fumarate reductase synthesis upon $H_2O_2$ formation.** Aerobic overnight cultures of LC106, LC126, LC114, LC128, and LC141 were grown with an additional catalase. Cells were then diluted into anaerobic minimal glucose (0.2%) and fumarate (40 mM) plus histidine or LBg and grown to log phase. Cultures of cells with the Frd plasmid (pH3) contained ampicillin. Cells were then assayed for $H_2O_2$ formation and Frd activity upon air exposure. Frd activity is normalized to the background strain and presented in parentheses above peroxide measurements. *, $p < 0.007$ *versus* background ($Hpx^-$).

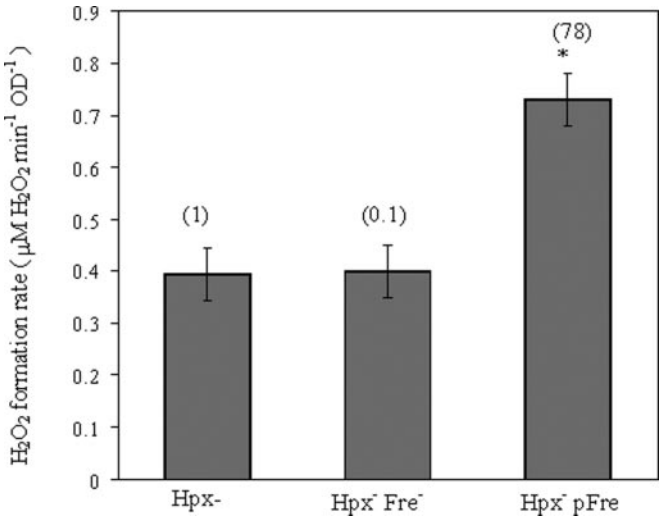


FIG. 5. **Overexpression of flavin reductase increases $H_2O_2$ formation.** Aerobic cultures of LC106, LC118, and LC110 were grown in LBg to log phase; at an $OD_{600}$ of 0.02, 0.4 mM isopropyl-β-D-thiogalactopyranoside was added to the strain containing the flavin reductase plasmid pFre (pFN3) to induce expression. At an OD of ~0.3, cells were assayed for flavin reductase (*Fre*) activity and a $H_2O_2$ formation rate. Flavin reductase activity is normalized to the background strain and presented in parentheses above the peroxide measurements. *, $p < 0.007$ *versus* background strain ($Hpx^-$).

Interestingly, an $Hpx^-$ $Frd^-$ mutant (LC126) also showed an increase in $H_2O_2$ production upon aeration. This result appeared contrary to the expected result, a decrease in $H_2O_2$ formation, if Frd were normally a substantial $H_2O_2$ source. Because Frd is a terminal electron acceptor in the respiratory chain, this increase may be due to a "back-up" of electrons onto a component upstream. This component could be NdhII, as was seen with the strain lacking quinone (Fig. 3). In sum, whereas Frd produced some $H_2O_2$ *in vivo*, it did so at substantial rates only when it was overproduced.

*Overproduction of Flavin Reductase Results in $H_2O_2$ Formation in Vivo*—The preceding experiments suggested that the primary $H_2O_2$ source might lie outside the respiratory chain. We looked, therefore, toward free flavins as a potential $H_2O_2$ source. Reduced free flavins, generated by flavin reductase, react with oxygen to produce $H_2O_2$ in vitro (46), presumably by same mechanism as the autoxidation of flavoenzymes. However, $Hpx^-$ cells lacking flavin reductase formed $H_2O_2$ at approximately the same rate as did the flavin reductase-proficient strain (Fig. 5). The $Hpx^-$ strain that overproduced flavin reductase 80-fold formed only 1.9-fold more $H_2O_2$ than did the $Hpx^-$ strain (Fig. 5). These data support the ability of free flavins to adventitiously make $H_2O_2$, but they indicate that flavin reductase is a relatively insignificant $H_2O_2$ source under these growth conditions.

Thus the three candidate enzymes NdhII, Frd, and flavin reductase generated varying amounts of $H_2O_2$ *in vivo*. However, they produced much less than did another unidentified source.

*Endogenous $H_2O_2$ Production during Growth on Different Carbon Sources*—The amount of oxidative stress that a bacterium experiences depends on the rates at which intracellular $O_2^-$ and $H_2O_2$ are produced. These rates should depend on the titers of auto-oxidizable enzymes in the cell, which, in turn, might change in response to growth on different carbon sources. To test this possibility we attempted to measure the rates of $H_2O_2$ production during log-phase growth on glucose, gluconate, glycerol, lactate, pyruvate, succinate, acetate, and casamino acids (2%). Respiration rates were measured in parallel with $H_2O_2$. $H_2O_2$ production rates varied modestly from one source to another (Table III). Unfortunately, these results were not very informative in suggesting the identity of the major $H_2O_2$ source.

Interestingly, whereas this strain could grow aerobically in liquid culture on fermentable carbon sources, it was unable to accommodate a transfer from an anaerobic glucose medium to aerobic media containing either succinate or acetate as the sole carbon source. No increase in $OD_{600}$ was seen in 20 h. In contrast, the $Hpx^+$ parental strain (MG1655) grew well with both. Notably, these two carbon sources are catabolized via the trichloroacetic acid cycle; the others can be catabolized via other pathways. Thus, the low level of $H_2O_2$ that accumulates in the $Hpx^-$ mutant is evidently sufficient to disrupt some catabolic activities. We note that this disruption could influence the rate of metabolic $H_2O_2$ production.

## DISCUSSION

*Autoxidation of Respiratory Enzymes*—Aerobic cells generate enough internal $H_2O_2$ to cripple themselves. Mutant strains of *E. coli* that are stripped of antioxidant defenses, that is, of the ability to scavenge endogenous $H_2O_2$ or, alternatively, to repair oxidized DNA, grow poorly or not at all in aerobic environments (14, 48–50). In this study we attempted to identify the mechanisms by which $H_2O_2$ forms inside *E. coli* and to determine whether the rate of $H_2O_2$ formation depends upon the identity of the growth substrate.

Our earlier work with respiratory vesicles *in vitro* had indicated, as shown in Reactions 1–3,

$$FADH^- + O_2 \rightarrow FADH^{\cdot} \sim O_2^-$$

REACTION 1

$$FADH^{\cdot} \sim O_2^- \rightarrow FAD^{\cdot} + H^+ + O_2^-$$

REACTION 2

$$FAD^{\cdot} + O_2 \rightarrow FAD^+ + O_2^-$$

REACTION 3

TABLE III
*$H_2O_2$ formation by an Hpx$^-$ strain grown on various carbon sources*

| Carbon source[a] | $t_D$[b] | $O_2$ consumption rate[c] | $H_2O_2$ formation rate[c] | $H_2O_2/O_2$ consumed |
|---|---|---|---|---|
| | *min* | *$\mu$M $O_2$ $s^{-1}$ $OD^{-1}$* | *$\mu$M $H_2O_2$ $s^{-1}$ $OD^{-1}$* | |
| Casamino acids | 48 | 2.4 | $8.3 \times 10^{-3}$ | $3.5 \times 10^{-3}$ |
| Glucose | 104 | 1.4 | $7.2 \times 10^{-3}$ | $5.1 \times 10^{-3}$ |
| Gluconate | 108 | 1.6 | $9.9 \times 10^{-3}$ | $6.2 \times 10^{-3}$ |
| Glycerol | 155 | 1.3 | $8.3 \times 10^{-3}$ | $6.4 \times 10^{-3}$ |
| Pyruvate | 112 | 1.5 | $4.8 \times 10^{-3}$ | $3.2 \times 10^{-3}$ |
| Lactate | 132 | 2.2 | $11.0 \times 10^{-3}$ | $5.0 \times 10^{-3}$ |
| Succinate | NG[d] | | | |
| Acetate | NG[d] | | | |

[a] Carbon source concentration, amino acid supplementation, and growth conditions are described under "Materials and Methods."
[b] $t_D$ = Doubling time in log phase.
[c] Measurements were done in duplicate and an average is presented.
[d] NG = No growth.

or in Reactions 4 and 5,

$$FADH^- + O_2 \rightarrow FADH^{\cdot} \sim O_2^{\cdot-}$$

REACTION 4

$$FADH^{\cdot} \sim O_2^{\cdot-} \rightarrow FAD^+ + H_2O_2$$

REACTION 5

that several dehydrogenases transfer electrons singly or sequentially to oxygen when it collides with their solvent-exposed reduced flavins (22). In those experiments, the greatest yield from the aerobic respiratory chain arose from NADH dehydrogenase II, and the greatest yield from the anaerobic chain arose from fumarate reductase. The autoxidation rate was greatest when the oxygen concentration was elevated or when oxidized quinone acceptors were unavailable so that the electrons were backed up onto the enzymes. Overproduction of either enzyme enhanced the yield of $H_2O_2$ or $O_2^{\cdot-}$.

The *in vivo* data of this study confirmed that NdhII and Frd can react with oxygen. There were several plausible reasons why it might have turned out otherwise. A fundamental concern was that, in our *in vitro* studies, rough handling during the preparation of either the vesicles or the purified enzymes might have caused some fraction of the enzymes to misfold and become artifactually vulnerable to oxidation. That was evidently not the case, because these enzymes turned out to autoxidize *in vivo* as well.

However, a more subtle issue is that the redox status of the enzymes and the distribution of electrons among the redox moieties within the enzymes are dependent both upon substrate concentrations and membrane potential. In fact, whereas the *in vitro* experiments had suggested that NdhII might generate 5–10 $\mu$M/s $H_2O_2$ *in vivo*, the present data shown that NdhII actually produced <1.5 $\mu$M/s. Why the difference? Right now we can only speculate. The steady-state redox status of the FAD moiety of NdhII reflects the dynamic balance between reduction by NADH, allosteric control by $NAD^+$, and oxidation by ubiquinone. Efforts were made in the *in vitro* experiments to replicate the physiological dinucleotide pools; however, differences between *in vitro* and *in vivo* pH, counterion concentrations, and protonmotive force may have altered these interactions. Similarly, because fumarate can block the reaction of Frd with oxygen (22, 33), the intracellular fumarate levels may have a big impact upon autoxidation rates.

*The Main Source of $H_2O_2$ Is Unknown*—Ultimately, this study has not answered a key question: what is the primary source of endogenous $H_2O_2$? Our results indicate that it lies outside the respiratory chain. This is a surprise, as the amenability of respiratory components to univalent redox reactions has always prompted workers to look there for biomolecules that can react with triplet oxygen. Formally, whenever we eliminated one respiratory enzyme, the electron flow could have been redirected to another one that generated $H_2O_2$ at the same rate. However, this idea is quantitatively improbable, because the rates at which flavoproteins react adventitiously with oxygen vary by orders of magnitude (33, 42). Thus, the replacement of a section of one redox chain with another is unlikely to precisely recreate the same flux of $H_2O_2$. Instead, we are inclined to believe that most $H_2O_2$ is formed when either a non-respiratory enzyme or a cellular metabolite reacts adventitiously with oxygen. The simplicity of this assay and the genetic tractability of *E. coli* give us hope that this experimental system will allow the question to be answered. This work is in progress.

*The Rate of $H_2O_2$ Formation Is Consistent*—Uncertainty has arisen regarding the rate at which mitochondria generate $H_2O_2$, with published values ranging from <0.15 to 2% of oxygen consumption (16, 51). The disparity has been variously attributed to the use of inhibitors (52), variability with different substrates (51), interference from endogenous peroxidases (25), and artifacts from the detection system (36). These problems have been circumvented with this *E. coli* system. Although we do not yet know the mechanism of its formation, with a variety of growth substrates $H_2O_2$ was produced in exponentially growing cells at rates between 0.3 and 0.7% of oxygen consumption and 9–22 $\mu$M/s inside the cell. Prior measurements of the intracellular scavenging activity had led to an estimate that these cells contain ~20 nM steady-state $H_2O_2$ (26). This value, then, is a quantitative measure of the $H_2O_2$ stress that growing cells experience. It is probable that the value differs in nutrient-starved cells.

*Acknowledgments*—We are grateful to the colleagues cited in Table I who provided strains for this work.

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