ultrafiltration unit with 100,000 molecular weight filter membranes. Metal samples were acidified with Ultrex HNO_3 (0.5 ml to 250 ml) and analyzed with model 975 Jarrel-Ash inductively coupled plasma (ICP) spectrophotometer. Lithium was also analyzed by flame atomic absorption spectrometry (Perkin-Elmer 2280 spectrophotometer). Anions were analyzed with a 2000i Dionex ion chromatograph. Organic carbon was measured with a Dohrmann carbon analyzer. Reagents for analysis of iron oxidation state by the 2,2'-bipyridine colorimetric method were added immediately after filtration to unacidified sample (*10*). Absorbance was measured within several hours for representative samples, and all samples were assayed within 2 days; the color development for the representative samples was stable over this period.

10. M. W. Skougstad *et al.*, *Techniques for Water Resources Investigations of the U.S. Geological Survey* (U.S. Geological Survey, Denver, 1986), Book 5, chapter A1, p. 387.
11. L. N. Plummer, B. F. Jones, A. H. Tresdell, *U.S. Geol. Surv. Water Res. Invest. 76-13* (1976).
12. J. I. Drever, *The Geochemistry of Natural Waters* (Prentice-Hall, Englewood Cliffs, NJ, 1982).
13. T. D. Waite and F. M. M. Morel, *J. Colloid Interface Sci.* **102**, 121 (1984).
14. ———, *Environ. Sci. Technol.* **18**, 860 (1984).
15. K. M. Cunningham, M. C. Goldberg, E. R. Weiner, *Photochem. Photobiol.* **41**, 409 (1985).
16. ———, in *Chemical Quality of Water and the Hydrologic Cycle*, R. A. Averett and D. M. McKnight, Eds. (Lewis Press, Ann Arbor, MI, 1987), p. 359.
17. E. L. Madsen, M. D. Morgan, R. E. Good, *Limnol. Oceanogr.* **31**, 832 (1986).
18. D. K. Norstrom, *U.S. Geol. Surv. Water-Supply Pap. 2270* (1985), pp. 113–119.
19. D. Dullin and T. Mill, *Environ. Sci. Technol.* **16**, 815 (1982).
20. Supported by the Hazardous Substances in Surface Waters Program of the U.S. Geological Survey–Water Resources Division. We acknowledge the sample collection efforts of D. H. Campbell, A. C. Duncan, R. M. Hirsch, C. L. Miller, B. D. Nordlund, B. Olver, R. C. Ruddy, N. E. Spahr, G. A. Wetherbee, and G. W. Zellweger, and comments on the manuscript by K. Nordstrom, D. Thorstenson, and D. Waite.

19 October 1987; accepted 10 March 1988

Toxic DNA Damage by Hydrogen Peroxide Through the Fenton Reaction in Vivo and in Vitro

JAMES A. IMLAY,* SHERMAN M. CHIN, STUART LINN†

Exposure of *Escherichia coli* to low concentrations of hydrogen peroxide results in DNA damage that causes mutagenesis and kills the bacteria, whereas higher concentrations of peroxide reduce the amount of such damage. Earlier studies indicated that the direct DNA oxidant is a derivative of hydrogen peroxide whose formation is dependent on cell metabolism. The generation of this oxidant depends on the availability of both reducing equivalents and an iron species, which together mediate a Fenton reaction in which ferrous iron reduces hydrogen peroxide to a reactive radical. An in vitro Fenton system was established that generates DNA strand breaks and inactivates bacteriophage and that also reproduces the suppression of DNA damage by high concentrations of peroxide. The direct DNA oxidant both in vivo and in this in vitro system exhibits reactivity unlike that of a free hydroxyl radical and may instead be a ferryl radical.

THE INSTABILITY OF PARTIALLY REduced oxygen species poses a serious threat to aerobic organisms. Consecutive univalent reductions of molecular oxygen to water produce three active intermediates, superoxide ($HO_2\cdot$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\cdot$):

$$O_2 \xrightarrow[H^+]{e^-} HO_2\cdot \xrightarrow[H^+]{e^-} H_2O_2 \xrightarrow[H^+]{e^-} HO\cdot + H_2O \xrightarrow[H^+]{e^-} 2H_2O \quad (1)$$

Scavenging enzymes, such as catalase, peroxidase, and superoxide dismutase (*1*), and DNA repair enzymes that correct oxidative lesions (*2*) are found throughout the aerobic biota. Despite the apparent significance of oxidative cell damage, the mechanism and the ultimate oxidant have not yet been established.

At least two mechanisms produce cell damage in the killing of *Escherichia coli* by exogenous H_2O_2 (*3*). Starved cells can tolerate considerable exposure to H_2O_2 before they are killed; this "mode-two" killing is due to uncharacterized cell damage and exhibits a classical multiple-order dose-response curve. In "mode-one" killing, actively growing cells are killed by lower, more physiological doses of H_2O_2, particularly if they lack enzymes required for recombinational or base-excision DNA repair pathways.

In the mode-one response the rate of killing is maximal at ~2.5 m*M* H_2O_2 but is roughly independent of H_2O_2 concentration and half-maximal between about 10 and 20 m*M* (Fig. 1). (This twofold difference in rate gives rise to large differences in survival as shown in Fig. 2A.) Prior exposure to high doses of H_2O_2 does not increase resistance to an immediate subsequent challenge with low doses (*3*), indicating that this is not a protective response. Similar dose-response curves to H_2O_2 are obtained when monitoring mutagenesis, the extent of lysogenic phage lambda induction, or postdamage filamentation of surviving cells (*4*), each of which is thought to be related to the extent of DNA damage. (These latter effects as well as killing are eliminated at low H_2O_2 concentrations if cells are starved before challenge.) Thus the unusual nature of the dose response would appear to reflect a characteristic dose response for the generation of DNA damage.

Because mode-one killing and the related phenomena occur only in actively metabolizing cells, available reducing equivalents might be essential to convert H_2O_2 into a toxic oxidant. *E. coli* can be made to accumulate reducing equivalents if respiration is inhibited with cyanide; when so treated, normally resistant DNA repair-proficient cells become extremely sensitive to mode-one killing by H_2O_2 (Fig. 2A). Cyanide exerts this effect by blocking respiration, since *ndh* mutants, which lack active NADH dehydrogenase (where NADH is the reduced form of nicotinamide adenine dinucleotide) and are also nonrespiring, are highly sensitive to mode-one killing but are not made appreciably more so by the addition of cyanide (Fig. 2, B and C).

The $HO\cdot$ radical, a highly reactive oxidant, has been implicated in peroxide-mediated oxidation of a variety of substrates (*5*). The univalent reduction of H_2O_2 was postu-

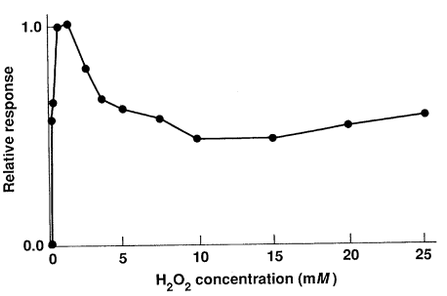

Fig. 1. Dependence of cellular responses on H_2O_2 concentration. The rate of killing of strain BW544 (*nfo xth*, defective in excision repair of oxidative DNA damage) is shown. Cells were exposed to H_2O_2 for 15 minutes and then plated. Colonies were counted after 24 to 48 hours. Virtually superimposable dose-response curves are obtained when scoring mutagenesis, induction of a phage lambda lysogen, or cell division delay (*4*). Many such curves were obtained with the maximum effect always between 1.5 and 2.5 m*M* H_2O_2. In this and subsequent figures, the results are those from a single experiment, representative of a minimum of three such experiments.

Department of Biochemistry, University of California, Berkeley, CA 94720.

*Present address: Department of Biochemistry, Duke University Medical Center, Durham, NC 27710.
†To whom correspondence should be addressed.

Table 1. Relative rates of reaction of various reductants with the immediate oxidant of *p*-NDA or phage lambda as determined by competition experiments. Competition curves like those of Fig. 4B were used to obtain relative reaction rates with the dye-bleaching and phage-inactivating oxidants. The reaction rates are relative to those between H_2O_2 and the indicated oxidants.

Reductant	Relative reactivity with		
	HO·	Oxidant of *p*-NDA	Oxidant leading to phage inactivation
p-NDA	439 (*5, 9*)	500	
Ethanol	63 (*13*)	60	$<10^{-4}$
Mannitol	63 (*13*)	51	10^{-3}
tert-Butanol	18 (*13*)	22	10^{-4}

lated by Fenton (*6*) and by Haber and Weiss (*7*) to explain the iron-dependent decomposition of H_2O_2 at acid *p*H. The metal acts as a redox catalyst of the catalase reaction (Eq. 5) through three reactions (Eqs. 2 through 4) known collectively as the Haber-Weiss cycle:

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + HO\cdot + H_2O \quad (2)$$

$$HO\cdot + H_2O_2 \rightarrow HO_2\cdot + H_2O \quad (3)$$

$$HO_2\cdot + Fe^{3+} \rightarrow O_2 + Fe^{2+} + H^+ \quad (4)$$

$$2H_2O_2 \xrightarrow{Fe} O_2 + 2H_2O \quad (5)$$

A variety of metal chelators can block the Fenton reaction (Eq. 2) by occupying metal coordination sites. Addition of dipyridyl to *E. coli* fully blocks mode-one killing by H_2O_2 (Fig. 3A). By contrast, mode-two killing is unaffected. Similar protection is conferred by *o*-phenanthroline and desferrioxamine mesylate. The mutagenesis and filamentation that accompany mode-one killing also do not occur if cells are challenged in the presence of these chelators.

The apparent involvement of both electrons and iron in mode-one killing suggests that the Fenton reaction might produce the DNA-damaging oxidant. Indeed, if this oxidant were HO· itself, then Eq. 3 of the Haber-Weiss cycle could account for the suppression of mode-one toxicity at 10 to 20 m*M* H_2O_2; that is, high concentrations of H_2O_2 could scavenge HO·. Moreover, since the reduction of iron is likely to limit the Fenton reaction in the cell, increasing the H_2O_2 concentration above ~1 m*M* would not increase the rate of Eq. 2 since Fe^{2+} would be limiting. However, one might expect the many endogenous reactants in the cell to mask the effect of millimolar concentrations of H_2O_2; more directly, 100 m*M* ethanol or mannitol, both of which have a greater ability than H_2O_2 to scavenge HO· radicals (Table 1), failed to protect against mode-one killing (Fig. 3B). A free HO· radical is thus unlikely to be the direct DNA oxidant.

We then studied the properties of Fenton reaction oxidants in vitro. In the simplest system, mixing of Fe(II) and H_2O_2 resulted in rapid Fe(II) oxidation and H_2O_2 decomposition. In excess H_2O_2, 1.8 moles of H_2O_2 were consumed for each mole of Fe(II) oxidized (*8*). [Fe(III) was not recycled under these conditions, presumably because of its precipitation at neutral *p*H as the hydroxide.] The inclusion of ethanol in this reaction changed the stoichiometry to one-to-one, indicating that ethanol competes to scavenge HO· formed by Eq. 2 before it can participate in Eq. 3:

$$HO\cdot + CH_3CH_2OH \rightarrow H_2O + CH_3\dot{C}HOH \quad (6)$$

The bleaching of *p*-nitrosodimethylaniline (*p*-NDA) has been used as another indicator of HO· (*9*). As expected from Eq. 2, a mixture of Fe(II) salts and H_2O_2 appeared to generate HO· that bleached the dye; moreover, the extent of bleaching was reduced at higher concentrations of H_2O_2 (Fig. 4A), presumably through Eq. 3.

Scavengers of the oxidant of the dye should quench its bleaching according to the function

$$\Delta A_0/\Delta A = 1 + K_I[I]/K_s[S] \quad (7)$$

where ΔA_0 is the extent of maximal bleaching, ΔA is the bleaching in the presence of inhibitor I, S is the substrate (*p*-NDA in this case), and K_I and K_s are the reaction rates of inhibitor and substrate, respectively, with the oxidant (*9*) (Fig. 4B). The relative abilities to scavenge the dye-bleaching oxidant calculated from such curves were indeed consistent with the identification of the oxidant of *p*-NDA as HO· (Table 1).

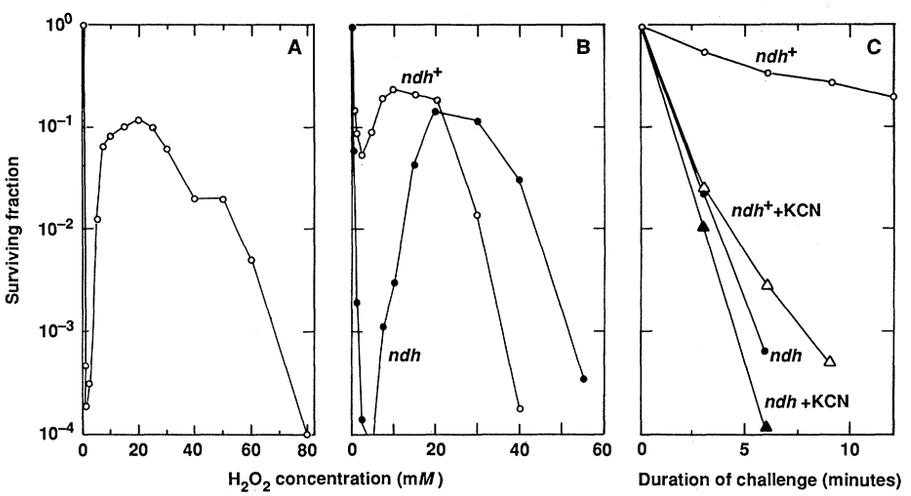

Fig. 2. Blocks in respiration sensitize normal bacteria to mode-one killing. (**A**) Potassium cyanide (3 m*M*) was added to strain AB1157 5 minutes before a 15-minute exposure to H_2O_2. In the absence of cyanide, approximately 60% of the cells survived the 2.5 m*M* H_2O_2 exposure (*3*). (**B**) Strains IY13 (*ndh*$^+$) and IY12 (*ndh*, lacking NADH dehydrogenase) were exposed to H_2O_2 for 15 minutes and then plated. (**C**) Strains IY13 and IY12 were challenged with 2.5 m*M* H_2O_2; at the times indicated, aliquots were withdrawn and plated to determine survival. Where indicated, 3 m*M* KCN was added 5 minutes before the challenge with H_2O_2.

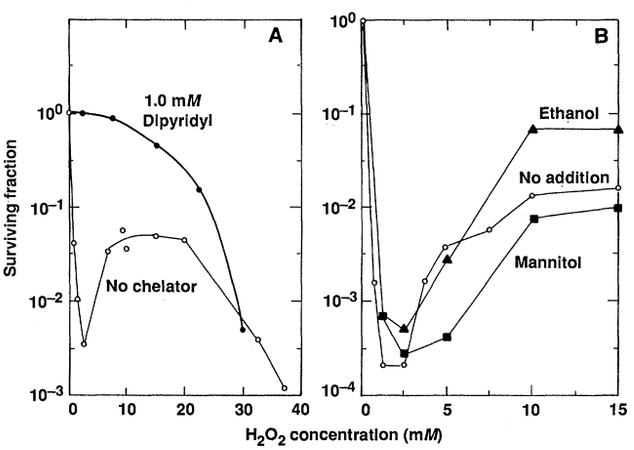

Fig. 3. Effects of inhibitors of the Haber-Weiss cycle on mode-one killing. (**A**) Strain BW9091 (*xth*) was exposed to H_2O_2 for 15 minutes and then plated. Where indicated, 1 m*M* dipyridyl was added to the cells 5 minutes before the challenge with H_2O_2. (**B**) Strain BW544 (*xth nfo*) was exposed to H_2O_2 for 4.5 minutes and then plated. Where indicated, 100 m*M* mannitol or ethanol was added to cells 10 minutes before challenge.

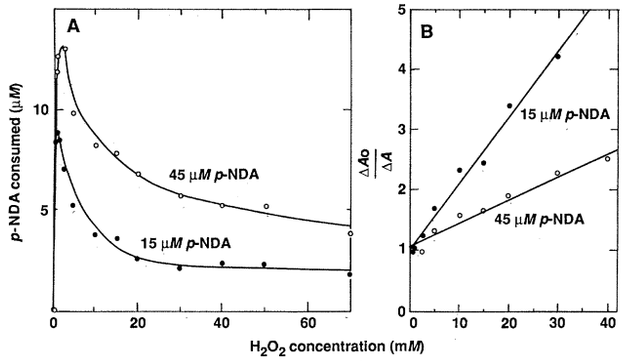

Fig. 4. High concentrations of H_2O_2 prevent dye bleaching by scavenging the oxidant of the dye. Reaction solutions contained 50 μ*M* $FeSO_4$, 45 μ*M* (○), or 15 μ*M* (●) *p*-nitrosodimethylaniline (*p*-NDA), and the indicated concentrations of H_2O_2. After 15 minutes, solutions were diluted 1:10, and the absorbance at 440 nm was determined. (**A**) Dye consumption. (**B**) Degree of bleaching. As noted in the text and in (*8*), data should fit Eq. 7. For this application, *I* is the amount of H_2O_2 in excess of that which produces that maximal level of bleaching. A least-squares fit has been plotted.

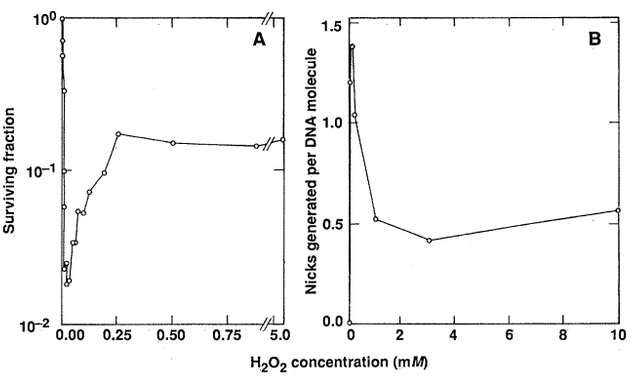

Fig. 5. DNA damage produced by Fenton reactions in vitro. (**A**) Lambda bacteriophage (10^5 plaque-forming units per milliliter) were mixed at room temperature with 0.8% NaCl, 0.5 μ*M* $FeSO_4$, and H_2O_2 as indicated. After 7.5 minutes, the phage were diluted, mixed with *E. coli* strain AB1157, and plated. Plaques were counted after 12 to 24 hours. (**B**) Phage PM2 DNA (17 μ*M* nucleotide concentration) was mixed with 0.8% NaCl, 10 m*M* ethanol, 80 n*M* $FeSO_4$, and H_2O_2 as indicated. After 7.5 minutes, catalase (2 μg/ml) was added, and DNA single-strand breaks were determined by a nitrocellulose filter binding assay (*14*). The data of both (A) and (B) represent reaction limits. The initial rates of iron oxidation and DNA damage are greater at higher H_2O_2 concentrations, as expected from Eq. 2.

In an attempt to relate the activity of the biological oxidant to the simple Fe(II)/H_2O_2 reaction, which generates a HO· radical that acts as a dye oxidant, we added bacteriophage lambda in place of the dye. Phage were inactivated in this system simultaneously with the oxidation of Fe(II); moreover, as with cell killing, at higher H_2O_2 concentrations the final extent of phage inactivation was reduced by roughly a factor of 2 (Fig. 5A). Phage inactivation did not occur if Fe(III) was substituted for Fe(II) or if metal chelators were included in the reaction. High concentrations of ethanol, mannitol, or *tert*-butanol were minimally effective at protecting the phage, and the relative efficiencies of H_2O_2 and other agents at scavenging the phage oxidant did not reflect their reaction rates with the HO·, indicating that free HO· was not the inactivating oxidant (Table 1).

Because strains of *E. coli* defective in DNA repair are particularly sensitive to mode-one killing and such strains accumulate single-strand breaks during exposure to H_2O_2 (*10*), we replaced intact phage with purified DNA in the in vitro system and measured the production of single-strand breaks in the DNA as a function of H_2O_2 concentration. Nanomolar amounts of iron were sufficient to generate detectable strand breaks, and the extent of DNA nicking was proportional to the iron concentration. Approximately half of the nicking could be prevented by micromolar amounts of ethanol at doses consistent with the scavenging of HO·. The remaining nicking was resistant to millimolar amounts of ethanol, and, when the H_2O_2 dose response was examined, this nicking was reduced (and remained roughly constant) at higher concentrations of H_2O_2 (Fig. 5B; compare with Fig. 1) (*11*). Thus a simple in vitro Fenton system reproduced the essential character of the dose response to H_2O_2 noted for the DNA-damaging process in vivo.

Although the Fenton reaction can generate scavengeable HO· radicals, an ethanol-resistant DNA oxidant is also produced in vitro and in vivo. In the organic bacteriophage or cell environments only the ethanol-resistant oxidant survives to cause detectable DNA damage. Studies of Fenton reactions in other systems have led to the postulation of a "crypto-hydroxyl radical" (*12*), a hydroxyl radical that derives distinct character from being complexed either to its precursive metal or to its substrate. In this way, the Fenton reaction (Eq. 2) might be postulated to include such an intermediate:

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+}\text{–}HO\cdot + H_2O \quad (8)$$

$$Fe^{3+}\text{–}HO\cdot \rightarrow Fe^{3+} + HO\cdot \quad (9)$$

$$Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + HO\cdot + H_2O \quad (2)$$

One might then propose that the biologically active radical would be the ferryl radical formed by Eq. 8, and that its breakdown (Eq. 9) would complete the Fenton reaction (Eq. 2) to produce the hydroxyl radical that bleaches *p*-NDA in an ethanol-sensitive reaction.

Ferrous atoms responsible for the DNA damage both in vitro and in vivo are probably complexed to the DNA rather than free in solution. Thus the inability of organic scavengers to inhibit DNA damage could be a consequence either of the chemical nature of the Fenton-generated radical or of the immediacy of its reaction with the DNA. Since the damage radical is prone to quenching by H_2O_2, the intermediacy of a nonhydroxyl radical, such as the ferryl radical, is indicated.

REFERENCES AND NOTES

1. I. Fridovich, *Science* **201**, 875 (1978).
2. B. Demple and S. Linn, *Nature (London)* **287**, 203 (1980); M. C. Hollstein, P. Brooks, S. Linn, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4003 (1984).
3. J. A. Imlay and S. Linn, *J. Bacteriol.* **166**, 519 (1986).
4. ———, *ibid.* **169**, 2967 (1987); S. Linn and J. A. Imlay, *J. Cell Sci. (Suppl.)* **6**, 289 (1987).
5. C. Walling, *Acc. Chem. Res.* **8**, 125 (1975).
6. H. J. H. Fenton, *J. Chem. Soc.* **65**, 899 (1894).
7. F. Haber and J. Weiss, *Proc. R. Soc. London Ser. A* **147**, 332 (1934).
8. For a combination of Eqs. 2 and 3 we would predict a ratio of 2:1. Should the $HO_2\cdot$ formed undergo dismutation to H_2O_2 and O_2, a ratio of 1.5:1 would be predicted.
9. W. Bors, C. Michel, M. Saran, *Eur. J. Biochem.* **95**, 621 (1979).
10. H. N. Ananthaswamy and A. Eisenstark, *J. Bacteriol.* **130**, 187 (1977); B. Demple, A. Johnson, D. Fung, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7731 (1986).
11. The subset of nicking sensitive to ethanol obeys the form of Eq. 7, with 50% inhibition by 25 μ*M* ethanol in competition with 67 μ*M* DNA nucleotide. These values are consistent with the reported reactivities of free HO· with ethanol (1.8×10^9 mol^{-1} sec^{-1}) and DNA nucleotide (3×10^9 to 8×10^9 mol^{-1} sec^{-1}) [F. Hutchinson, *Prog. Nucleic Acid Res. Mol. Biol.* **32**, 115 (1985)]. By contrast, the ethanol-resistant nicking requires >10 m*M* ethanol for 50% inhibition.
12. W. Bors, M. Saran, G. Czapski, *Dev. Biochem.* **11B**, 1 (1980); E. F. Elstner, *Annu. Rev. Plant Physiol.* **33**, 73 (1982); R. J. Youngman, *Trends Biochem. Sci.* **9**, 280 (1984).
13. J. D. Rush and W. H. Koppenol, *J. Biol. Chem.* **261**, 6730 (1986).
14. U. Kuhnlein, E. E. Penhoet, S. Linn, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1169 (1976).

15. Supported by NIH grants GM19020 and P30ES01896 and an NSF graduate fellowship to J.A.I.

3 November 1987; accepted 18 February 1988