Oxygen toxicity in *Treponema pallidum*: deoxyribonucleic acid single-stranded breakage induced by low doses of hydrogen peroxide

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The effect of hydrogen peroxide on *Treponema pallidum* was investigated. The *in vitro* loss of virulence (as measured by rabbit oculation) of *T. pallidum* was accelerated by as low as 100 μ*M* hydrogen peroxide in the complex maintenance medium used. igher doses led to rapidly accelerated death with 500 μ*M* hydrogen peroxide causing sterilization of the medium within 3 to 4 h. nce hydrogen peroxide is known to cause single-stranded breaks in DNA, the effect of hydrogen peroxide on the treponemal nome was examined. Extensive breakage was caused by 100 μ*M* hydrogen peroxide as determined on alkaline sucrose adients. A limit was reached at 250 μ*M* and above. Single-stranded breaks could be demonstrated as early as 5 – 10 min after posure to hydrogen peroxide when the treponemes were exposed to 250 μ*M* hydrogen peroxide; accelerated death was evident 2 h past exposure demonstrating that DNA breakage was preceding death. Treponemal death caused by penicillin did not result DNA breakage. The repair-proficient bacterium *Escherichia coli* K-12 was compared with *T. pallidum*. It required 10 – 100 nes more hydrogen peroxide to cause various levels of breakage. *Escherichia coli* K-12 rapidly repaired DNA breakage once ydrogen peroxide was removed by addition of catalase. *Treponema pallidum*, in comparison, showed little or no repair *in vitro*. ddition of catalase or dithiothreitol to the medium protected against all but a low level of breakage; this may reflect on the ability catalase and reducing agents to protect *T. pallidum* against oxygen toxicity *in vitro*.

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On a vérifié l'effet du peroxyde d'hydrogène sur *Treponema pallidum*. *In vitro*, dans un milieu de culture complexe de conservation, une concentration de peroxyde d'hydrogène aussi basse que 100 μ*M* accélère la perte de virulence de *T. pallidum* (après inoculation au lapin). Des doses plus élevées de 500 μ*M* de peroxyde entraînent une mort rapide et le milieu devient stérile en moins de 3 à 4 h. On sait que le peroxyde est connu pour causer des bris de l'ADN monocaténaire et on a décidé d'étudier l'effet du peroxyde d'hydrogène sur le génome du tréponème. Tel que mesuré sur gradient de sucrose alcalin, une concentration de 100 μ*M* de peroxyde d'hydrogène cause des bris importants, mais une limite est atteinte à 250 μ*M* et plus. Lorsque les tréponèmes sont exposés à 250 μ*M* de peroxyde d'hydrogène, des bris des brins monocaténaires sont visibles dès les 5 à 10 min qui suivent l'exposition au peroxyde. La mort accélérée devient évidente environ 2 h après le contact confirmant ainsi que le bris de l'ADN précède la mort. Lorsque la mort des tréponèmes est causée par la pénicilline il n'y a pas de bris de l'ADN. Une souche d'*Escherichia coli* K-12 capable de réparer ce type de dommage a été comparée à *T. pallidum*. Il faut 10 à 100 fois plus de peroxyde d'hydrogène pour causer différents niveaux de bris. *Escherichia coli* K-12 répare rapidement le bris de l'ADN dès que le peroxyde d'hydrogène disparaît par addition de catalase. Comparativement *T. pallidum* démontre peu ou pas de réparation *in vitro*. L'addition de catalase ou de dithiothreitol au milieu protège contre tous les types de bris mais avec une faible efficacité. Cette observation peut se refléter sur la capacité de la catalase et des agents réducteurs à protéger *T. pallidum* contre la toxicité de l'oxygène *in vitro*.

[Traduit par le journal]

Introduction

Although *Treponema pallidum* is thought to require oxygen for active metabolism (Barbieri and Cox 1981; Lysko and Cox 1978), it is rapidly killed by oxygen *in vitro* (Fitzgerald 1981). Recent work has shown that toxic oxygen reduction products appear to mediate this killing with both hydrogen peroxide and the hydroxyl radical involved (Steiner *et al.* 1984). *Treponema pallidum* is not known to synthesize any oxygen protective enzymes (Austin *et al.* 1981; Steiner *et al.* 1984); thus, *T. pallidum* should be highly susceptible to these reactive molecules. While the intracellular target for oxygen toxicity in *T. pallidum* has not been defined, numerous targets for oxygen toxicity exist in the cell. Hydrogen peroxide (H_2O_2) is known to cause single-stranded breaks in DNA in bacteria (Ananthaswamy and

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Eisenstark 1977) and in mammalian cells (Bradley and Erickson 1981), and hyperbaric oxygen causes depletion of ribosomes (Harley *et al.* 1981) and peroxidation of lipids in *Escherichia coli* (Harley *et al.* 1978). Single-stranded breaks in DNA are induced by such diverse factors as high temperature (Ahmad *et al.* 1978), ionizing radiation (Town *et al.* 1973), and toxic oxygen products (Ananthaswamy and Eisenstark 1977; Brawn and Fridovich 1981). Normal aerotolerant bacteria such as *E. coli* have effective DNA repair enzyme systems which prevent these breaks from becoming lethal (Willets and Clark 1969). Mutants are known for several of these repair pathways and they are highly sensitive to agents which cause chromosomal breakage (Howard-Flanders and Theriot 1966; Willets and Clark 1969; Kapp and Smith 1970) including oxygen (Morimyo 1982). At least one fastidious microbe, *Mycoplasma gallisepticum*, has no known repair systems, so even the wild-type strains are rapidly killed when their DNA is damaged with ultraviolet light (Ghose *et al.* 1977). In this paper we show that the high sensitivity of *T. pallidum* to hydrogen peroxide appears to be related to DNA damage and that effective repair of DNA could not be demonstrated *in vitro*. (This work was presented in a preliminary form at the 12th International Union for Biochemistry Meeting in Perth, Western Australia, August 13–18, 1982).

Materials and methods

Maintenance and harvesting of T. pallidum

Treponema pallidum was maintained by intratesticular inoculation in adult rabbits as previously described (Graves *et al.* 1975).

Survival assays of T. pallidum

After the majority of rabbit tissue cells had been removed by centrifugation (1000 × *g* for 10 min), the treponemes were incubated in Eagle's minimal essential medium (EMEM) with 10% fetal calf serum at 34°C. Appropriate amounts of H_2O_2 were added from a 50 m*M* stock to give the experimental concentrations. At appropriate intervals thereafter treponemes were tested for motility and virulence in rabbits. These assays have been described previously (Graves *et al.* 1975). The relationship between the time of appearance of a syphilitic lesion on the shaved back of a rabbit and the number of viable treponemes injected into the rabbit was taken from a previously established standard curve (Graves *et al.* 1975). Although individual rabbit variation occurred in the time of onset of lesions, the differences between the various experimental rabbit groups were highly reproducible and statistically significant.

Labelling of cells and alkaline sucrose gradient ultracentrifugation

Treponema pallidum were harvested from infected rabbit testes as soon as an orchitis became evident. The procedure for harvesting *T. pallidum* has been described in detail previously (Steiner *et al.* 1981). Harvesting medium was EMEM without serum. Rabbit cells were removed by centrifuging twice for 10 min at 1000 × *g*. Microscopic examination showed less than 4 × 10^2 mammalian cells/mL after this procedure consisting mainly of erythrocytes and spermatozoa when samples were examined by dark-field microscopy. Yield of *T. pallidum* was 3–5 × 10^7 treponemes/mL in all experiments; labelling was not performed if the yield was lower.

After removal of the eucaryotic cells from the EMEM, fetal calf serum (10%) and dithiothreitol (0.5 m*M*) were added. The dithiothreitol was necessary to consume excess oxygen in the unreduced medium. [^{3}H]Uridine (20 μCi/mL) (1 Ci = 37 GBq) or [^{3}H]adenine (20 μCi/mL) were then added and the tubes (Bellco anaerobic tubes) were flushed for 60 s with sterile, oxygen-free nitrogen to lower the dissolved oxygen level, and tightly stoppered. This procedure was necessary to maintain viability for more than a few hours. After 24 h incubation at 34°C, the incorporation was terminated, at which time treponemal survival (as measured by culture motility) was still 85–100%. The treponemes were then removed from the labelling medium by centrifuging at 12 000 × *g* for 20 min using an SS34 head in an RC2-B Sorvall centrifuge. The pelleted treponemes were then resuspended in fresh EMEM medium containing 10% fetal calf serum and 20 m*M* HEPES buffer; in most experiments the fresh medium contained no dithiothreitol (DTT). DTT reacts rapidly with oxygen reduction products such as hydrogen peroxide and protects against its destructive effect on *T. pallidum* DNA (see Results). Although excision repair and subsequent survival is enhanced by inhibiting replicative DNA synthesis (Swenson 1976), we were required to use this medium because *T. pallidum* survival was too unreliable when the experiment was performed in buffer; untreated control survival was often very short in buffer. To make conditions comparable, *E. coli* was also treated with H_2O_2 and rejoining of single-stranded breaks studied in growth medium instead of in the more customarily used buffers which do not support replicative DNA synthesis.

The culture was then divided into 1-mL aliquots and each treated differently. Various amounts of a 50 m*M* stock of H_2O_2 were added to all but the control sample. At the end of a 15-min exposure, the H_2O_2 was inactivated by addition of excess catalase (600 units/mL), the samples were placed on ice, and immediately layered onto preformed sucrose gradients. This amount of catalase was sufficient to destroy all of the hydrogen peroxide in the 500 μ*M* exposed culture within 45 s. Controls consisted of samples of treponemes which were treated identically to the experimental samples except for the exposure to hydrogen peroxide. In two experiments an extra control was performed which consisted of a sample that was exposed to 1000 units/mL of penicillin for 2 h to cause treponemal death. This control was used to demonstrate that the DNA breakage seen in these experiments was a consequence of hydrogen peroxide exposure and not DNA degradation owing to cell death. Penicillin's known mode of action involves interaction with the cell wall; since it has no direct affect on bacterial DNA, it makes an appropriate control for nonspecific DNA breakage related to treponemal death instead of breakage owing to the inducing treatment. After 2 h of exposure to this very high level of penicillin, treponemal motility fell rapidly. Longer exposures to penicillin were not used since nonspecific

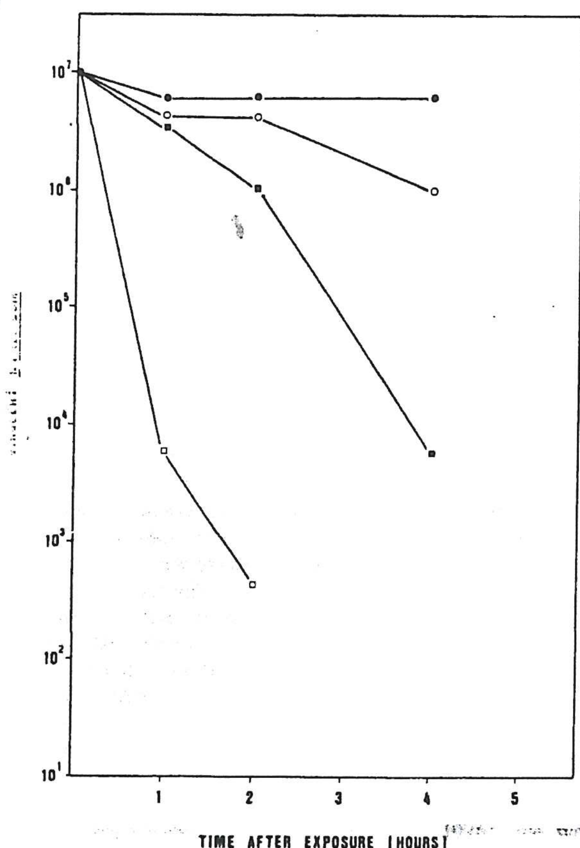

FIG. 1. Effect of hydrogen peroxide on the survival of *T. pallidum* as measured by virulence for rabbits by intradermal injection. Number of surviving *T. pallidum* was determined from a previously published standard curve (Graves *et al.* 1975). Survival was tested in $EMEM_{III}$ medium with 20 m*M* HEPES, no DTT. ●, Control, not exposed to added hydrogen peroxide; ○, 100 μ*M* hydrogen peroxide (final concentration) added to medium; ■, 250 μ*M* hydrogen peroxide (final concentration) added to medium; □, 500 μ*M* hydrogen peroxide (final concentration) added to medium. No virulent *T. pallidum* remained in the 500 μ*M* hydrogen peroxide exposed culture beyond 2 h.

degradation of DNA can occur when bacterial cells are held for extended periods after loss of viability. After the samples were layered on the gradients, they were allowed to sit at 23°C in the dark for 30 min to allow for lysis of the treponemes and disassociation of the DNA (lysis occurred within the first 1 – 2 min). Treponemal DNA was analyzed for single-stranded breakage using the alkaline sucrose gradient method of McGrath and Williams (1966) with a few modifications, as indicated below. The composition of the gradients was as described by Ananthaswamy and Eisenstark (1977) with the following modification. The gradient consisted of 17 mL, and 0.20 mL of sample was then centrifuged for 2 h at 25 000 rpm using an SW 27.1 rotor in a Beckman model L2-65B ultracentrifuge. Thirty samples were collected by puncturing the bottom of the nitrocellulose tube, and the samples were allowed to stand for at least one additional hour to eliminate any residual labelled RNA (Norris *et al.* 1980). The DNA was precipitated onto glass-fiber filters with 5% trichloracetic acid (TCA) in a VFM3 vacuum filtration manifold (Amicon Corp., Lexington, MA, U.S.A.). The filtrates were washed with water and methanol before counting. After drying overnight at 34°C, the glass-fiber filters were placed in poly Q vials (Beckman, U.S.A.) and 7.5 mL of scintillation fluid added. The scintillation fluid consisted of 5 g of 2,5-diphenyloxazole (PPO) and 0.4 g of 1,4-bis-2(4-methyl-5-phenyl-oxazolyl) benezene (dimethyl POPOP) per litre of toluene. The samples were each counted for 10 min in a Packard model 2002 Tri Carb liquid-scintillation counter. In experiments with *E. coli* K-12, the bacteria were grown overnight in an M9 salts medium (Ananthaswamy and Eisenstark 1977) with 0.1% glucose and 1.0% casamino acids. The cells were resuspended to approximately 5×10^6/mL in M9 medium with 15 μCi/mL [^{3}H]thymidine and 250 μg/mL deoxyadenosine (Boyce and Setlow 1962). After labelling, the *E. coli* cells were treated exactly the same as *T. pallidum*. The number average molecular weight (NAMW) of the DNA fragments was calculated using the formula of Ley (1973). Coli phage T2, grown on *E. coli* B, and labelled with [^{3}H]thymidine, was used to calibrate the sucrose gradient (Burgey and Hershy 1963). Labelled T2 phage were purified from the lysate and *E. coli* debris was removed by centrifugation at 12 000 × *g* for 20 min using an SS34 head in a RC2-B Sorvall centrifuge. The virus in the supernatant was purified using the method of Yamamoto *et al.* (1970).

To determine if single-stranded breaks in treponemal DNA could be repaired, the cells were reincubated after catalase inactivation of the H_2O_2 under a 100% nitrogen atmosphere to stop any further potential damage from oxygen reduction products. Motility after the 15-min exposure to 100 μ*M* H_2O_2 was still 70% or greater (data not shown). The cells were reincubated at 34°C for periods of 30 and 60 min, respectively, at which time samples were withdrawn for analysis by placing them directly into the lysis buffer on top of the alkaline sucrose gradients. *Escherichia coli* K12 was reincubated at 37°C aerobically after catalase addition and samples withdrawn for centrifugation at 7.5 and 15 min after H_2O_2 inactivation to determine the amount of repair of alkaline-labile breaks.

Results

Survival of T. pallidum *exposed to hydrogen peroxide*

Treponema pallidum rapidly lost virulence on addition of low levels of H_2O_2 to EMEM containing 10% fetal calf serum. Toxicity was repeatedly seen with 100 μ*M* H_2O_2 and levels of 250 μ*M* or greater led to rapid sterilization of the culture (Fig. 1). Previous data (Steiner *et al.* 1984), using motility as an index of survival, has shown that toxicity for treponemes can be detected at concentrations of H_2O_2 as low as 50 μ*M*. The toxicity of hydrogen peroxide was completely eliminated by including excess catalase in the medium (Steiner *et al.* 1984). Based on this data, the level of tolerance to H_2O_2 of *T. pallidum* is similar to that of most obligate anaerobes (McLeod and Gordon 1923).

TABLE 1. Single-stranded breaks induced in *T. pallidum* and *E. coli* DNA by various doses of hydrogen peroxide

Treatment	No. of single-stranded breaks per genome	
	T. pallidum	*E. coli* K-12
50 μM H_2O_2	33	—
100 μM H_2O_2	44	—
250 μM H_2O_2	TNTD	—
500 μM H_2O_2	TNTD	33
1 mM H_2O_2	—	33
5 mM H_2O_2	—	132

NOTE: *Treponema pallidum* and *E. coli* K-12 were treated with the indicated dose of hydrogen peroxide for 15 min in normal survival or growth medium. *Treponema pallidum* was resuspended in fresh medium before exposure to remove any residual DTT. Single-strand breaks calculated as indicated in Materials and methods. Results are averages of two to five experiments. TNTD, too numerous to determine by alkaline sucrose sedimentation. H_2O_2 was inactivated with excess catalase (600 units/mL final concentration) before it was layered on the alkaline sucrose gradients.

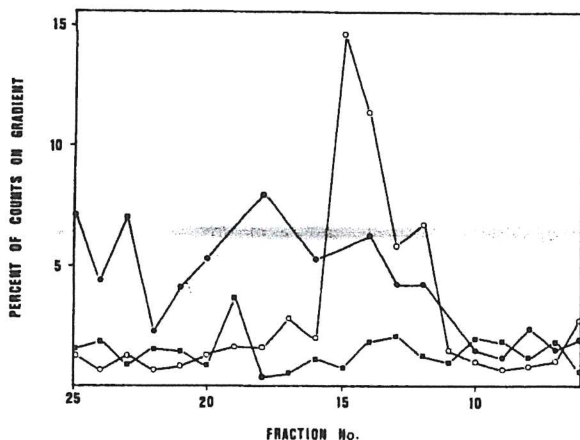

FIG. 2. DNA breakage induced in *T. pallidum* by a 15-min exposure to various amounts of hydrogen peroxide. Unexposed control DNA shows a broad peak with a NAMW of 350 × 10^6; treatment with 100 μM hydrogen peroxide lowered the NAMW to 125 × 10^6. Treatment with 500 μM hydrogen peroxide gave essentially the same results as with 250 μM hydrogen peroxide. *Treponema pallidum* was labelled with [^{3}H]adenine as described in Materials and methods. ●, Untreated control; ○, exposed to 100 μM hydrogen peroxide for 15 min; ■, exposed to 250 μM hydrogen peroxide for 15 min.

Single-stranded DNA breakage resulting from hydrogen peroxide treatment

Treatment of *T. pallidum* with H_2O_2 resulted in extensive breakage of the cells' DNA and collapse of the chromosome (Fig. 2, Table 1). Damage occurred at 100 μM H_2O_2 and extensive degradation of DNA appeared to result from exposures to 250 μM H_2O_2 (Fig. 2). In *E. coli* K-12, minor damage was seen at 500 μM H_2O_2 and

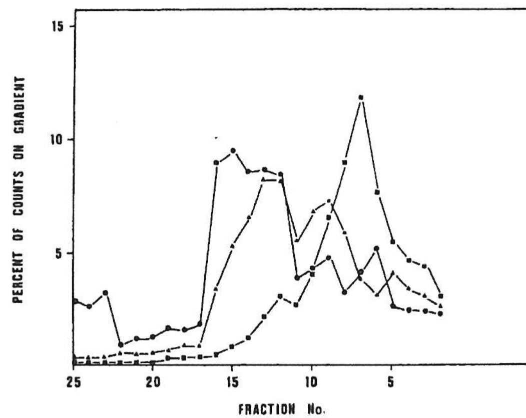

FIG. 3. DNA breakage induced by exposure of *E. coli* to 5 mM hydrogen peroxide and subsequent repair. *Escherichia coli* was labelled with [^{3}H]thymidine as described in Materials and methods and then exposed to 5 mM hydrogen peroxide for 15 min. At this time the hydrogen peroxide was inactivated by the addition of 600 units/mL of catalase. The culture was then incubated at 37°C for 15 min to allow for repair of single-stranded DNA breaks. ●, Untreated control; ■, 5 mM hydrogen peroxide exposed culture immediately after inactivation of hydrogen peroxide with catalase; ▲, 5 mM hydrogen peroxide exposed culture 15 min after inactivation of hydrogen peroxide with catalase. Note that most of the single-stranded breaks have been repaired within 15 min.

continued to increase up to 5 mM, the highest concentration tested (Fig. 3, Table 1). Far less extensive breakage was seen at 5 mM H_2O_2 with *E. coli* than with *T. pallidum* at 250 μM. The sensitivity of *T. pallidum* was similar to that of repair-defective mutants of *E. coli* treated with DNA-damaging chemicals (Town *et al.* 1973). *Treponema pallidum* showed two important properties that were similar to repair-deficient strands of *E. coli*: the extremely low doses of hydrogen peroxide which could cause DNA breakage and the inability to limit degradation as seen by the loss of high molecular weight DNA when exposed to rapidly, lethal doses of hydrogen peroxide (see Discussion). In experiments performed using a 250 μM H_2O_2 treatment, alkaline-labile breaks were seen 5 – 10 min after treatment (data not shown). As can be seen in Fig. 1, it took up to 2 h before the damage resulted in highly significant loss of viability, indicating that DNA breakage preceded loss of viability. Killing the treponemes by exposing them to 1000 units/mL of penicillin for 2 h resulted in no detectable DNA degradation (Fig. 4), making it unlikely that the DNA breakage seen on exposure to H_2O_2 was caused by nonspecific DNA degradation related to loss of viability by other causes.

Substantial protection against DNA breakage was provided by including catalase in the medium (Fig. 5). When 750 units/mL of catalase was included in the

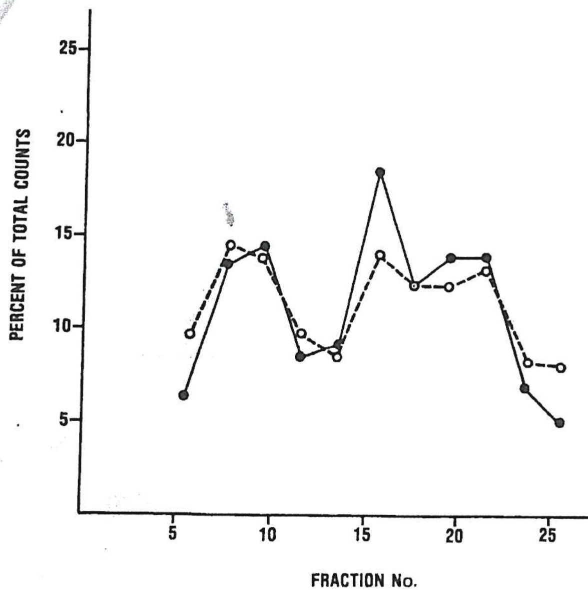

FIG. 4. Lack of DNA single-stranded breakage in *T. pallidum* exposed to 1000 units/mL of penicillin for 2 h. The majority of the *T. pallidum* were immotile after the exposure period. ●———●, Control, no penicillin in culture; ○---○, 1000 units/mL of penicillin in culture for 2 h before extraction of *T. pallidum* DNA.

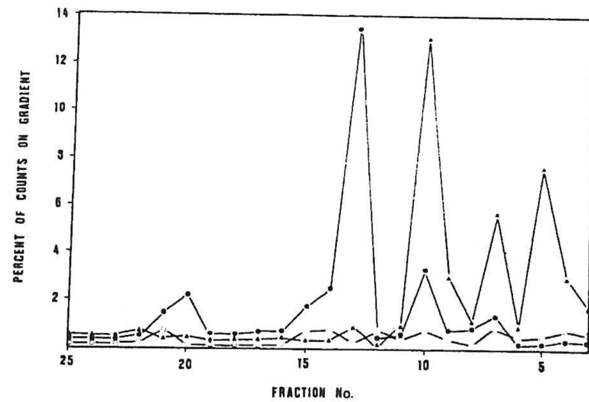

FIG. 5. Partial protection against DNA single-stranded breakage in *T. pallidum* by 750 units/mL of catalase. *Treponema pallidum* DNA was labelled with [^{3}H]uridine as described in the Materials and methods, and then exposed to 250 μM hydrogen peroxide. This dosage normally results in complete loss of high molecular weight DNA from exposed *T. pallidum*. Catalase provided significant but only partial protection against single-stranded DNA breakage (see Results and Discussion). ●, Untreated control; ○, *T. pallidum* culture exposed to 250 μM hydrogen peroxide for 15 min before inactivation of hydrogen peroxide with catalase; ▲, *T. pallidum* culture containing 750 units/m*L* of catalase exposed to 250 μM hydrogen peroxide for 15 min.

medium, partial protection against DNA breakage occurred (Fig. 5). In all three catalase protection experiments performed, some breakage of treponemal DNA did occur, so protection was not complete. Catalase, which cannot penetrate the treponemal cell, gives far less protection against oxygen toxicity than thiols which can penetrate the cell (Steiner *et al.* 1984); thus, hydrogen peroxide which has escaped destruction long enough to penetrate the treponemal cell could be responsible for catalase giving only partial protection against DNA damage induced by hydrogen peroxide. Note that in Fig. 5 the labelling of *T. pallidum* DNA was performed with [^{3}H]uridine. Uridine is incorporated to a lesser extent than adenine in *T. pallidum* (Norris *et al.* 1980) and the peak is far less broad owing to this fact. The calculated NAMW was identical using either precursor. Multiple peaks were seen in the catalase-protected treponemes in each of the experiments with most of the single-stranded DNA similar or identical to the untreated DNA. The extra peaks varied between experiments both as to number and location on the gradient – indicating that at least a low level of damage had occurred to part of the population of treponemes even after this short exposure to low levels of hydrogen peroxide (the 750 units/mL of catalase was sufficient to destroy the added H_2O_2 completely within 20–30 s).

When 1 m*M* DTT was present in the medium instead of catalase, DNA breakage was largely eliminated (Fig. 6), both from exposures to 100 and 250 μM H_2O_2. This may reflect the greater protective capacity of reducing agents compared with oxygen-detoxifying enzymes (Steiner *et al.* 1984).

Repair of hydrogen peroxide induced single-stranded breaks in the DNA of T. pallidum *and* E. coli *K-12*

Treatment of *T. pallidum* with 100 μM H_2O_2 resulted in 30–60 single-stranded DNA breaks per genome (Table 1). The ability of *T. pallidum* to repair single-stranded breaks induced by 100 μM H_2O_2 was substantially less than that of *E. coli* exposed to 5 m*M* hydrogen peroxide which induced about twice as many breaks (Table 1). *Escherichia coli* rapidly repaired breaks in its DNA; by 15 min after inactivation of the hydrogen peroxide by catalase it had repaired at least 75% of the breaks observed at 0 time (Table 2, Fig. 3). In contrast, *T. pallidum* showed little or no repair of the lower level of breaks induced by 100 μM hydrogen peroxide at either 30 or 60 min post-exposure. In two experiments no repair was seen; in the third approximately 25% of the breaks were repaired after 30 min but no increase was seen at 60 min after the inactivation of the hydrogen peroxide. The number of breaks actually increased slightly in the other experiments at both 30 and 60 min after inactivation of the hydrogen peroxide (Table 2). Treponemal motility when the experiment was terminated was around 50%, having fallen from 80–95%

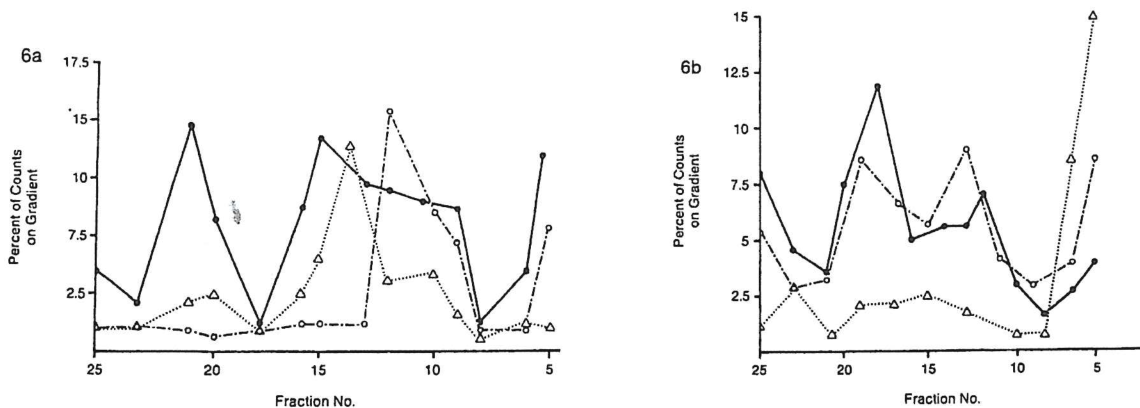

FIG. 6. Effect of 1 m*M* dithiothreitol on single-stranded DNA breakage induced by hydrogen peroxide. *Treponema pallidum* were labelled and treated as previously but suspended in medium containing 1 m*M* dithiothreitol. The treponemes were treated (a) with 100 and (b) with 250 μ*M* hydrogen peroxide. After a 15-min exposure, residual hydrogen peroxide was destroyed by treating the culture with 600 units/mL of catalase and the DNA was analyzed as described in Materials and methods. Note that high molecular weight DNA is retained in the presence of reducing agents. ●, *Treponema pallidum* DNA, no treatment; ○, *T. pallidum* DNA treated with (a) 100 μ*M* or (b) 250 μ*M* hydrogen peroxide in the presence of 1 m*M* dithiothreitol; Δ, *T. pallidum* DNA treated with (a) 100 or (b) 250 μ*M* hydrogen peroxide; no dithiothreitol in incubation medium.

TABLE 2. Comparison of the repair of single-stranded breaks induced by hydrogen peroxide in the DNA of *T. pallidum* and *E. coli*

		No. of single-stranded breaks per genome (% repaired)	
Treatment	Time (min)	*T. pallidum*	*E. coli* K-12
100 μ*M* H_2O_2	0	47	—
	30	67 (0%)	—
	60	85 (0%)	—
5 m*M* H_2O_2	0	—	132
	7.5	—	69 (48%)
	15	—	33 (75%)

NOTE: *Treponema pallidum* and *E. coli* were treated with the indicated dose of hydrogen peroxide in the same manner as in Table 1. After inactivation of the hydrogen peroxide by excess catalase (600 units/mL final concentration), the cultures were returned to the incubator (34°C for *T. pallidum*, 37°C for *E. coli* K12) and samples withdrawn at the indicated times. *Treponema pallidum* were maintained under nitrogen during the period allowed for repair.

before exposure to H_2O_2. In Fig. 7, a comparison of the repair capacities of *T. pallidum in vitro* and *E. coli* is shown. The data is the combined results from two experiments each and demonstrates the poor capacity of *T. pallidum* to repair single-stranded DNA breaks *in vitro* compared with a repair-proficient bacterium such as *E. coli*.

Discussion

The ability of a microorganism to survive and grow in the presence of oxygen is dependent on a number of factors. Survival is especially dependent on a variety of enzymes which act to detoxify oxygen reduction products (Fridovich 1978). Oxygen reduction products result from aerobic respiration and can rise to very high levels in organisms which do not produce detoxifying enzymes which act to detoxify oxygen reduction products (Fridovich 1978). An example is *Lactobacillus plantarum* which produces up to 4 m*M* hydrogen peroxide in the culture medium when grown aerobically (Archibald and Fridovich 1981). Since hydrogen peroxide can cause breakage of DNA (single or double stranded) (Ananthaswamy and Eisenstark 1977), deficiency in DNA repair mechanisms greatly increases susceptibility to hydrogen peroxide (Ananthaswamy and Eisenstark 1977; Carlsson and Carpenter 1980; Morimyo 1982). We believe this to be a possible explanation for the intolerance of *T. pallidum* to high levels of oxygen.

The ability to repair DNA is of great importance to the survival of an organism and involves a minimum of three enzyme systems in aerobic organisms (Hanawalt *et al.* 1979). Damage to DNA by reactive molecules, such as hydrogen peroxide, can take many forms. Likewise, ionizing radiation, an analogous system which has been much more intensively studied, not only causes direct breakage of the DNA helix but also causes base and sugar damage (Town *et al.* 1973). Modified purines and pyrimidines can lead to apurinic or apyrimidinic lesions in the chromosome which must be repaired. Hydrogen peroxide has been reported to cause the loss of adenine from DNA (Uchida *et al.* 1965). Loss of adenine leads to single-stranded breakage of DNA (Ljungquist 1977; Gossard and Verley 1978) which is repaired by repair system *I* in *E. coli* (Hanawalt *et al.* 1979; Witkin 1976). The most plentiful DNA polymerase found in *E. coli*

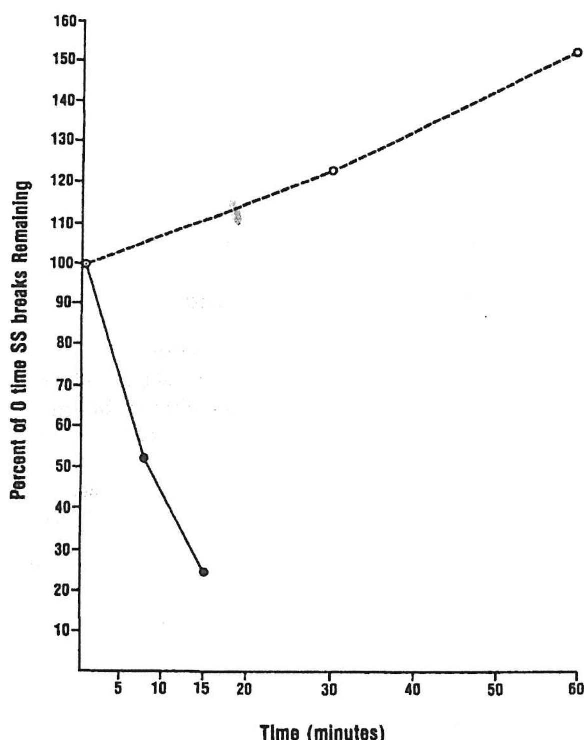

FIG. 7. Comparison of the repair of alkaline-labile breaks in DNA induced in *E. coli* and *T. pallidum* by hydrogen peroxide. *Escherichia coli* was treated with 5 m*M* hydrogen peroxide and *T. pallidum* with 100 μ*M* hydrogen peroxide, each for 15 min. The hydrogen peroxide was inactivated with 600 units/mL catalase and repair of alkaline-labile breaks was followed. Note lack of repair in *T. pallidum* despite approximately 70% motility at start of the experiments. Composite of two experiments with each organism. ○, *Treponema pallidum*; ●, *E. coli*. SS, single-stranded.

(Kornberg 1969) is involved in this system; this repair system is totally independent of DNA replication and proceeds in buffer as rapidly as in growth medium (Witkin 1976). Many of the DNA repair functions are inducible in *E. coli* K-12 and are under the control of the rec A and lex A gene products (Kenyon and Walker 1980). *Escherichia coli* mutants occur with defects in the major DNA repair functions (Howard-Flanders and Theriot 1966); these mutants illustrate the importance of the repair functions to survival since 50% of rec A and 75% of rec BC mutant cultures consist of nonviable bacteria (Capaldo and Barbour 1975). In the case of rec A mutants, the nonviable bacteria in the culture contain no DNA since they are unable to limit DNA degradation once the chromosome has been damaged (Capaldo and Barbour 1975). It has been suggested that lesions occur naturally during the synthesis of DNA and that repair enzymes are necessary to maintain the chromosome integrity even under normal growth conditions (Capaldo and Barbour 1975). Also, recent work has shown that a pol A rec B double mutant was inviable because of an enormously increased sensitivity to oxygen toxicity; at the nonpermissive temperature of 43°C viability was increased at least six logs by incubating under anaerobic conditions (Morimyo 1982). The importance of oxygen toxicity in relation to DNA repair is emphasized by the fact that in *E. coli* there appears to be an inducible repair system, related to the ionizing radiation repair system, which is induced by hydrogen peroxide (Demple and Halbrook 1983).

It now appears likely that *T. pallidum* does not contain any of the major oxygen-detoxifying enzymes (Austin *et al.* 1981; Steiner *et al.* 1984). However, *T. pallidum* also appears to require oxygen to carry out active metabolism (Barbieri and Cox 1981; Norris *et al.* 1980). Interestingly, one other group of bacteria show these characteristics, the mycoplasmas. Although an obligate aerobe, *Mycoplasma pneumoniae* contains no superoxide dismutase or catalase (Lynch and Cole 1980). One of the smallest mycoplasmas, *M. gallisepticum*, does not repair ultraviolet (UV) induced DNA lesions (Ghose *et al.* 1977). Based on our data, the ability to repair DNA lesions appear to be either absent or very limited in *T. pallidum*. *Treponema pallidum* was exceptionally sensitive to the formation of alkaline-labile sites in its DNA after treatment with hydrogen peroxide. As little as 100 μ*M* hydrogen peroxide caused damage to the DNA; *E. coli* K-12 required at least 10 times as much hydrogen peroxide to cause the same level of damage. Hydrogen peroxide damage to DNA preceded loss of viability and a close correlation between DNA damage and subsequent cell death was seen. The treponemes showed little ability to repair hydrogen peroxide induced breaks during *in vitro* incubation. Whereas *E. coli* efficiently repaired the majority of breaks induced by 5 m*M* hydrogen peroxide within 15 min, *T. pallidum* was unable to repair the lower level of DNA breaks induced by a concentration of 100 μ*M* hydrogen peroxide during an incubation of 1 h after inactivation of the hydrogen peroxide by catalase. We saw little or no repair of DNA breaks induced by 100 μ*M* hydrogen peroxide in our experiments *in vitro*. No data is available on whether *T. pallidum* could possibly have greater DNA repair capacities *in vivo*. Single-stranded breaks are predominately repaired by direct ligase action or excision-repair mechanisms (Town *et al.* 1973; Swenson 1976), both of which repair mechanisms occur in the absence of replicative DNA synthesis. Thus, the absence of effective mechanisms of repair of single-stranded breaks under nongrowing *in vitro* conditions suggest that *T. pallidum* may not have very efficient DNA repair mechanisms. The *T. pallidum* response to DNA damage was very similar to the repair-defective mutants of *E. coli* discussed above, and showed extreme sensitivity to

a DNA damaging treatment (hydrogen peroxide), an apparent inability to limit DNA degradation as seen by the loss of high molecular weight DNA after treatments with lethal doses of hydrogen peroxide, and the absence of DNA repair under the conditions used in these experiments.

A reduced ability to repair hydrogen peroxide induced DNA damage would put severe restraints on the growth potential of *T. pallidum* and may be the major factor in the inability to sustain its growth *in vitro*. Inefficient repair of single-stranded breaks would also probably increase the likelihood of lethal double-stranded breaks. Repair of double-stranded breaks is very dependent on multiple genome copies being present in the bacterium (Krasin and Hutchinson 1981). Bacteria with long generation times, like *T. pallidum* (Fitzgerald 1981), generally only have a single copy per cell, so double-stranded breaks could be assumed to be lethal.

The lack of DNA repair capacities *in vitro* and high sensitivity to genome damage by oxygen metabolites may explain the obligate requirement for reducing agents by *T. pallidum in vitro* (Fitzgerald 1981). Reducing agents are able to chemically repair DNA breaks induced by radiation (Ginsberg and Webster 1969; Sawada and Okada 1970). Radiation induced breaks are largely the product of oxygen metabolities such as hydrogen peroxide and the hydroxyl radical (Parshad *et al.* 1982). Evidence exists that exogenously added dithiothreitol is able to penetrate *T. pallidum* membranes and enter the cell (Fitzgerald *et al.* 1980); thus, its protective effect may be due to its ability to chemically repair oxygen-induced damage inside the treponemal cell.

The high sensitivity of *T. pallidum* to genome damage may also partially explain its obligately parasitic life. Protection from oxygen reduction products by host tissues is probably of primary importance during the period of active bacterial multiplication in host tissue. Consumption of oxygen by the actively growing tissues in which *T. pallidum* normally grows (Fitzgerald 1981; Steiner *et al.* 1983) and detoxification of oxygen reduction products by tissue enzymes, provides an attractive niche for an organism capable of penetrating host connective tissue. Once large-scale multiplication of the treponemes had occurred, the presumed toxic effect of large numbers of treponemes on mammalian cells (Fitzgerald *et al.* 1981; Oakes *et al.* 1982; Wong *et al.* 1983) would attract phagocytic cells to the site of infection. Activated macrophages secrete large amounts of hydrogen peroxide (Nathan *et al.* 1979) which may be lethal for *T. pallidum in situ*. Thus, there is a strong possibility that the elimination of *T. pallidum* from the primary focus of infection, where it is initially growing but from which it subsequently disappears, may also be related to its intolerance to hydrogen peroxide.

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