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Effects of Chloramine on *Bacillus subtilis* Deoxyribonucleic Acid

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The lesions induced in *Bacillus subtilis* deoxyribonucleic acid (DNA) after treating bacterial cells (in vivo) and bacterial DNA (in vitro) with chloramine were studied biologically and physically. Single-strand breaks and a few double-strand scissions (at higher chloramine doses) accompanied loss of DNA-transforming activity in both kinds of treatments. Chloramine was about three times more efficient in vitro than in vivo in inducing DNA single-strand breaks. DNA was slowly chlorinated; the subsequent efficiency of producing DNA breaks was high. Chlorination of cells also reduced activity of endonucleases in cells; however, chlorinated DNA of both treatments was sensitized to cleavage by endonucleases. The procedure of extracting DNA from cells treated with chloramine induced further DNA degradation. Both treatments introduced a small fraction of alkali-sensitive lesions in DNA. DNA chlorinated in vitro showed further reduction in transforming activity as well as further degradation after incubation at 50 C for 5 h whereas DNA extracted from chloramine-treated cells did not show such a heat sensitivity.

A great deal of work has been carried out to investigate the mode of chlorine disinfection; however, much less attention has been given to the mutagenic potentiality of this disinfectant. W. C. Boyle (Ph.D. thesis, California Institute of Technology, Pasadena, 1963) demonstrated the chemical reaction between chloramine (NH_2Cl) and pyrimidines, and that the reactivity of cytosine is higher than that of uracil and thymidine. The possibility that deoxyribonucleic acid (DNA) is the site of chlorination has been raised by Prat et al. (12, 13); they obtained an appreciable quantity of 5-chlorocytosine and 5-chlorouracil from an acid hydrolysate after sodium hypochlorite (NaOCl) treatment of bacterial DNA and ribonucleic acid (RNA), and cytosine was chlorinated more than uracil and thymidine. Evidence of incorporation of chlorine into DNA was also obtained by Eisenstark and Riva (also working in this laboratory; unpublished data); after treating calf thymus DNA with $NaOCl^{36}$, radioactive chlorine remained in acid-precipitable material. Hayatsu et al. (6) have reported a rapid decrease in the absorbance of ultraviolet light of both calf thymus DNA and yeast transfer RNA after NaOCl treatment. They also found that after treatment of cytosine an intermediate, 4N,5-dichlorocytosine, was generated which was readily converted into 5-chlorocytosine on treatment with hydrochloric acid. Further aspects of the action of hypochlorous acid (HOCl) on cytosine under different physiological conditions have been reported by Patton et al. (11). The inhibiting effect of chlorine on biological activity of DNA was observed by Hsu (7) using transforming DNA of *Haemophilus influenza* pretreated with chlorine. The preliminary results of Eisenstark (personal communication) have shown that *Bacillus subtilis* DNA was also reduced in its transforming activity after treatment with chloramine and hypochlorous acid and that the sodium hypochlorite-treated DNA yielded broken strands.

The above studies have been performed by treating the nucleic acids and nucleic acid bases with chlorine. The present study addressed the question of whether chlorine treatment of living cells could involve the modification and the destruction of the genetic material.

MATERIALS AND METHODS

Bacterial strains. All strains used in this article were derivatives of indole-requiring *B. subtilis* strain 168 (14).

Cell preparation. Cells grown overnight in 5 ml of Penassay broth were centrifuged and suspended in 50 ml of Spizizen minimal medium [per liter contains: $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 14 g; Na citrate, 1 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g] supplemented with 0.5% glucose, 0.05% casein hydrolysate, and 25 μg of those nutrients required for growth of auxotrophic

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strains per ml. Thymine-requiring strains were used for DNA labeling experiments, and additional 0.35 to 0.4 mg of thymidine and 10 μCi of ^{14}C per 0.19 μmol of thymidine or 0.1 mCi of ^{3}H per 0.07 μmol of thymidine were added to 50 ml of growth medium. Cells were grown at 37 C until the start of stationary phase and were then centrifuged and washed twice with 0.05 M phosphate buffer, pH 7, suspended in 12 ml of phosphate buffer, and were either used immediately or stored in liquid nitrogen after adding dimethyl sulfoxide to a final concentration of 5%.

DNA preparation. [^{3}H]DNA used for chloramine treatment was extracted from *B. subtilis* cells strain SB747 *(hisB tryC thy)* at log phase of growth. Cells were lysed in SSC (0.15 M NaCl plus 0.015 M sodium citrate) with 12% sucrose and 1 mg of lysozyme per ml at 37 C for 30 min. After adding ribonuclease (RNase), 50 μg/ml, the suspension was incubated at 37 C for 3 h. The lysis was completed by addition of 25% sodium lauryl sulfate to a final concentration of 0.5% and incubated at 70 C for 20 min. Pronase was then added to the lysate (1 mg/ml final concentration) and the lysate was incubated at 37 C for 3 h. At the end of the incubation, 5 M $NaClO_4$ was added to the lysate to a final concentration of 1 M. An equal volume of chloroform and one-fifth volume of *n*-octanol were added and the lysate was shaken at room temperature for 30 min. After spinning the lysate at 27,000 $\times$ *g* for 15 min the top layer was precipitated by cold ethanol and the precipitate was dissolved in 0.1$\times$ SSC-0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4. RNase and Pronase treatments were repeated after the addition of one-tenth volume of 10$\times$ SSC. The solvent was deproteinized with chloroform-*n*-octanol three more times. The cold-ethanol-precipitated DNA was again dissolved in the same buffer (10 ml) and was then dialyzed against two changes of 1 liter of the same buffer. DNA concentration was determined by absorption at 260 and 280 nm.

Chloramine treatment. HOCl was diluted in phosphate buffer to different concentrations. NH_2Cl was formed by incubating eight parts of HOCl with one part of 0.1 M NH_4Cl (in vivo treatment) or 1 M NH_4Cl (in vitro treatment) at 37 C for 1 h: $HOCl + NH_4Cl \rightarrow NH_2Cl + H_2O + HCl$ (1). The prepared cells or DNA was diluted 10 times into the NH_2Cl solution to a final concentration of 10^8 to 4×10^8 cells/ml or 11 to 22 μg of DNA/ml and treated for 30 min at 37 C. The reaction was stopped by adding 1 volume of 0.02 M (in vivo treatment) or 0.1 M (in vitro treatment) sodium thiosulfate ($Na_2S_2O_3$). In each experiment, the highest dose of NH_2Cl was added to the corresponding amount of thiosulfate before the addition of the cells or DNA and was used as control.

Viable cell counts were scored on plates of nutrient agar (Difco).

Transformation. Transforming DNA was prepared from the chloramine-treated and control cells as described, but without the 70 C treatment and without further treatments after the DNA was first dissolved in 0.1$\times$ SSC. The method of preparing the competent cells (SB202 *aroB hisB trpC tyrA*) and the procedure for transformation were described by Stewart (15). The media used to select the *aroB*$^+$, *hisB*$^+$, *trpC*$^+$, and *tyrA*$^+$ transformants contain all amino acids (25 μg/ml) except phenylalanine, histidine, tryptophan, and tyrosine, respectively. The media selected for tyrosine transformants were supplemented with 25 μg of shikimic acid per ml.

The viable cell counts and the colony counts in transformation were obtained by averaging colony counts of two plates. The minimum number of colonies per plate counted was 107 and the maximum standard deviation in colony counts was $\pm$12%.

Sucrose gradients. Cell lysate (0.15 to 0.30 μg of DNA) or purified DNA (0.15 to 1.1 μg) was layered on top of a 5 to 20% linear sucrose gradient (neutral, 1 M NaCl, 0.01 M Tris-hydrochloride, pH 8; alkaline, 0.8 M NaCl-0.2 M NaOH, pH 11.8). [^{3}H]P22 DNA (a gift from V. Sgaramella) was used as standard either in the same gradient with the sample or in a parallel gradient in the same centrifuge run. The gradients were centrifuged at 20 C for 1.5 to 4 h at 35,000 to 40,000 rpm in the IEC model SB-405 rotor. Fractions were collected dropwise from holes drilled in the bottom of the tubes directly onto Whatman GF/C filter disks. The disks were dried and the ^{3}H or ^{14}C activities were counted in a liquid scintillation counter.

The molecular weights of DNA were calculated from the formula (5): $D_1/D_2 = (M_1/M_2)^{\alpha}$. D_1 and D_2 are the distances sedimented by sample and standard, and M_1 and M_2 are the respective molecular weights. The distances were based on the mean position of the material in the gradient and therefore express average molecular weights only. Values of 27.0×10^6 and 13.5×10^6 for whole molecules of P22 DNA at neutral and alkaline gradients, respectively, were used as standard molecular weights. The α value is 0.35 for DNA in neutral gradients, 0.40 for DNA in alkaline gradients, and 0.549 for the denatured DNA in neutral gradients (18).

DNA uptake by competent cells. A 0.9-ml amount of SB202 competent cell suspension was incubated with 0.1 ml of the [^{3}H]DNA solutions to be tested at the DNA concentration of 0.45 μg/ml. At the conclusion of a 30-min incubation period, 0.05 ml of a 1-mg/ml of solution of deoxyribonuclease (DNase) (in 1 mg/ml bovine serum albumin-0.1 M Tris, pH 7.0) was added to the mixture of cells and the suspension was incubated at 37 C for 30 min. As a control, separate portions of the DNA solutions were pretreated for 30 min at 37 C with 0.1 ml of 100 μg of DNase per ml (in 100 μg/ml of bovine serum albumin-10^{-1} M $MgCl_2$-0.1 M Tris, pH 7.0) and then 0.9 ml of cell suspension was added. The difference between the ^{3}H counts fixed when DNase treatment of the DNA followed or preceded incubation of the cell suspension with DNA was taken as representing the DNA taken up. After incubation the cells were either filtered through membrane filters (Millipore Corp.), washed with Spizizen solution and counted, or used for the determination of transformants.

RESULTS

Effects of chloramine treatment in vivo on DNA-transforming activity and DNA molecu-

lar weight. The survival of *B. subtilis* populations treated with increasing concentrations of chloramine and the transforming activity of DNA extracted from these cells are shown in Fig. 1. (To simplify the discussion, DNA extracted from treated bacteria will be called V-DNA; DNA treated in vitro will be called T-DNA. "Chlorinated" means treated with chloramine. We do not have new data here on the introduction of chlorine atoms into DNA.) V-DNA does show a reduction in transforming activity for the markers tested, *tyrA*$^+$ and *hisB*$^+$. Further studies showed some impairment of uptake of V-DNA, compared to control, but not enough to account for the total reduction in genetic activity (Table 1). It seems that although part of the V-DNA can be taken up by the competent cells, the lesions induced prohibit the DNA from participating further. It was also found, from the same experiment, that the cotransfer of the linkage group (*aroB*$^+$ *hisB*$^+$ *tyrA*$^+$) is reduced in V-DNA. This indicates that reduction in biological activity of DNA was accompanied by the introduction of DNA strand breaks.

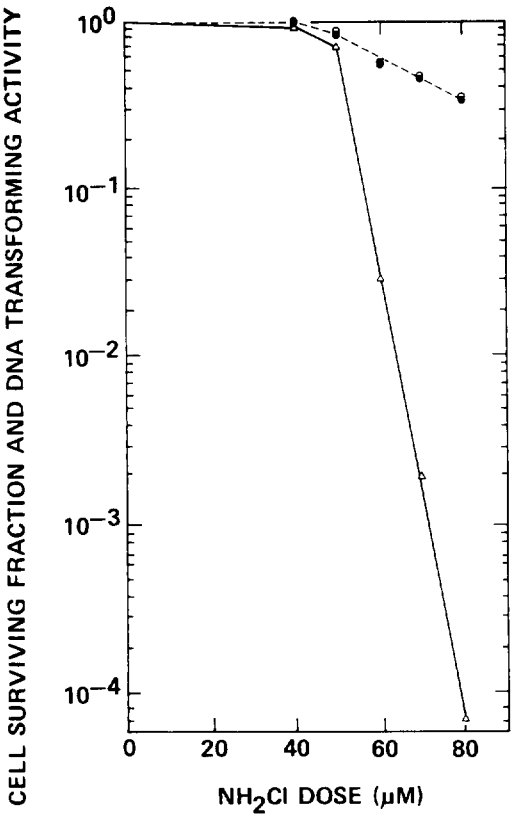

FIG. 1. *Cell surviving fraction and DNA-transforming activity of B. subtilis after chloramine treatment. Cells of strain 168 (trpC hisB*$^+$ *tyrA*$^+$*) were treated with different doses of* NH_2Cl. *After treatment, an equal amount of SB156 cells (trpC*$^+$ *hisB tyrA) was added into each sample. DNA was extracted from the mixed cells and served as donor to the recipient cells, SB202 (aroB trpC hisB tyrA). The trpC*$^+$ *transformants were used as internal controls. The ratios of hisB*$^+$ *or tyrA*$^+$ *transformants to trpC*$^+$ *transformants were the indices to the DNA activity of the 168 cells. Concentration of DNA was 0.2 to 0.25 μg/ml during transformation. Symbols: (△) surviving fraction; (○) hisB*$^+$*-transforming activity; (●) tyrA*$^+$*-transforming activity.*

Physical evidence that chloramine treatment of cells leads to single-strand breaks in DNA is obtained by zone sedimentation. Figure 2 shows that alkaline-denatured V-DNA is degraded from 65 to 44 or 14 mega D (mega D, 10^6 average molecular weight unless otherwise stated). In neutral gradients the shift is only from 140 to 137 or 105 mega D. These data point out that many single-strand breaks introduced in DNA by chloramine treatment are exposed by denaturation with alkali; further double-strand scissions occur at higher chloramine doses. In this experiment, 56 and 112 μM chloramine treatments give nonadditive results by producing 0.5 and 3.6 breaks per 65 mega D single-strand DNA, respectively. [To simplify the calculation, it is assumed that DNA breaks evenly, i.e., hits = (average molecular weight of control/average molecular weight of treated) − 1]. In a separate experiment, by treating the cells from the same culture with 28, 56, and 84 μM chloramine, 0, 0.6, and 2.0 breaks per 65 mega D single-strand DNA, respectively, were induced. If the two experiments are combined, the chloramine effect seems to show an initial lag, but may be more nearly additive in the range from 56 to 112 μM. It is presumed that some chloramine is taken up by other structures in the cells before it can reach the DNA molecules in sufficient concentration to induce the breaks.

The induction of DNA breaks is also time dependent. Figure 3 demonstrates that the longer the treatment, the slower the sedimentation of DNA. The number of DNA breaks per single-strand 66 mega D molecule induced are 1.3, 2.0, and 3.1 for 10-, 20-, and 40-min treatment, respectively.

Whether the addition of sodium thiosulfate also stops further degradation of DNA was investigated. After 10 min of chloramine treatment at 37 C, sodium thiosulfate was added to the chlorinated bacterial samples. DNA of the chlorinated cells incubated further at 37 C has a molecular weight of 26 mega D compared with 29 mega D of sample kept in ice. Sodium thio-

TABLE 1. *Effects of chloramine treatment in vivo on DNA-transforming activity and DNA uptake*[a]

NH_2Cl (μM)	Cell survival (%)	Single marker transforming activity (%)			Linked markers co-transforming activity (%)		DNA uptake (%)
		aroB$^+$	*hisB*$^+$	*tyrA*$^+$	*aroB*$^+$ *hisB*$^+$ *tyrA*$^-$/ *tyrA*$^+$	*aroB*$^+$ *hisB*$^+$ *tyrA*$^+$/ *hisB*$^-$	
0	100	100	100	100	77.50	66.50	100
56	32.50	69.42	67.77	66.67	69.50	61.50	91.01
112	0.00026	12.60	9.82	11.82	58.00	50.50	53.72

[a] [^{3}H]DNA was extracted from SB566 cells (*trpC thy*) after NH_2Cl treatment in vivo. SB202 competent cell suspension was used to test transforming activity and uptake of the [^{3}H]DNA solutions. Cotransformation was performed by replicating 200 *hisB*$^+$ and 200 *tyrA*$^+$ transformants from each treatment to phenylalanine-, histidine-, and tyrosine-deficient media.

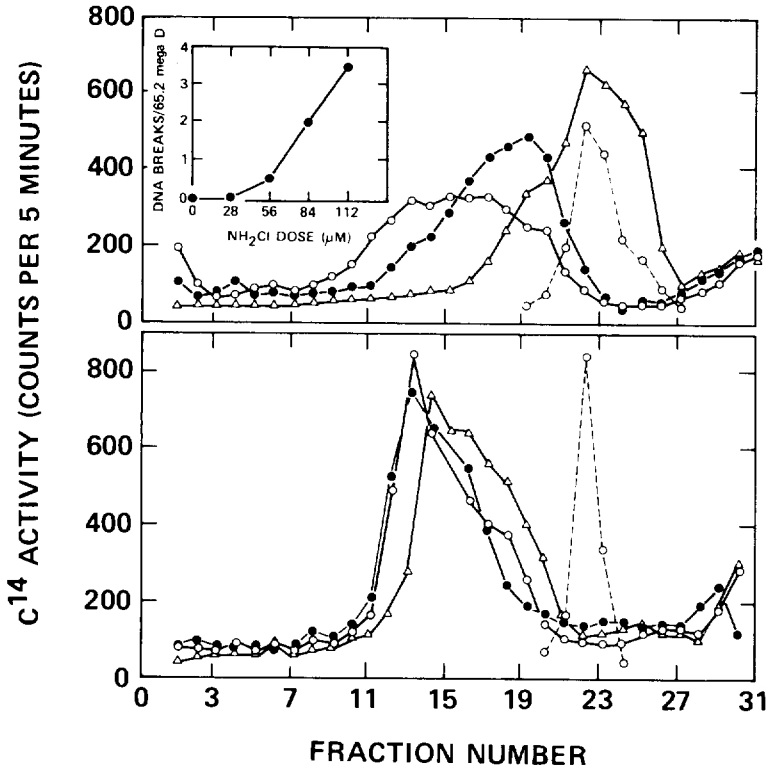

FIG. 2. *Degradation of DNA in vivo with different chloramine doses. The [^{14}C]thymidine-labeled cells SB566 (trpC thy) were treated with NH_2Cl and lysed by lysozyme (1 mg/ml) in SSC at 37 C for 30 min at cell concentrations less than 4 × 10^8/ml. The lysates were added to 0.05% SLS, incubated at 37 C until clear, and then layered on 5 to 20% sucrose gradients for zone centrifugation. Average molecular weights for 0- (–○–), 56- (●), 112- (△) μM NH_2Cl treatments were 65, 44, and 14 mega D, respectively, for alkaline gradients and 140, 137, and 105 mega D, respectively, for neutral gradients. (top) Alkaline gradients, insert shows DNA breaks per single strand 65 mega D molecule as a function of NH_2Cl doses; (bottom) neutral gradients; (- -○- -), P22 DNA.*

sulfate stops most of the DNA degradation in chlorinated cells; however, small portion of degradation is present after neutralization (Fig. 3).

Effect of chloramine treatment in vitro on DNA molecular weight. The reaction of chloramine with DNA (in vitro) was also studied. The sedimentation patterns of T-DNA resemble

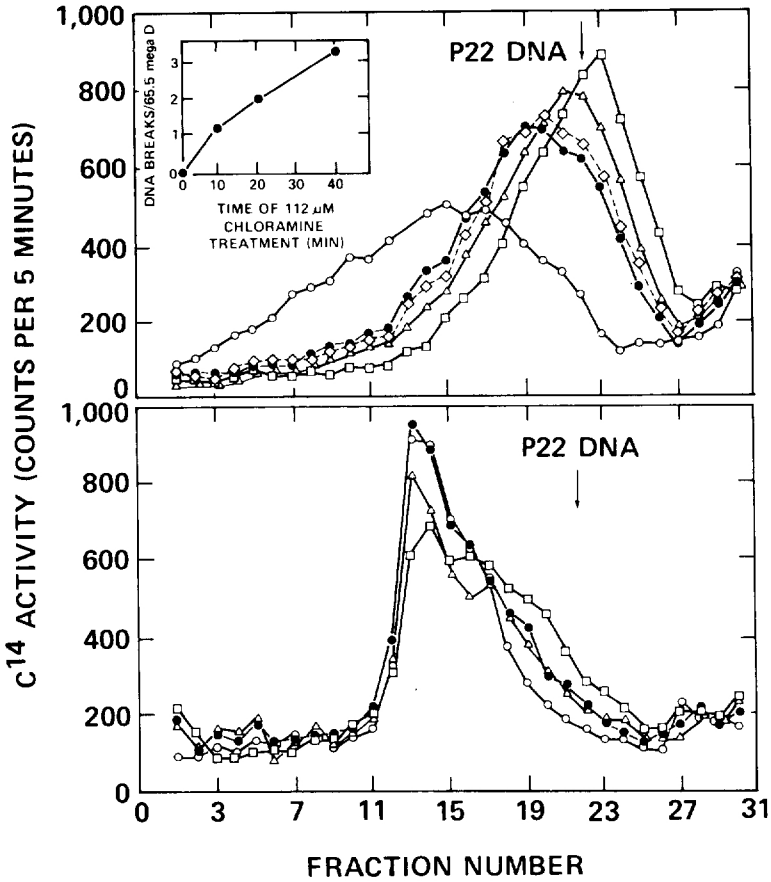

FIG. 3. *Degradation of DNA by chloramine in vivo with different treatment time. SB747 cells (trpC hisB thy) labeled with [^{14}C]thymidine were treated with 112 μM chloramine. Samples were removed at different intervals and neutralized with equal volumes of 0.02 M sodium thiosulfate and kept in ice. All the samples were lysed as described in Fig. 2. Average molecular weights for 0- (○), 10- (●), 20- (△), and 40- (□) min treatments were 66, 29, 22, and 16 mega D, respectively, for alkaline gradients and 150, 137, 133, and 112 mega D, respectively, for neutral gradients. One sample (10-min treatment with NH_2Cl) was kept in a 37 C water bath for 30 min after the addition of sodium thiosulfate (◇) and its DNA average molecular weight was 27 mega D in alkaline gradient. (top) Alkaline gradients, insert shows DNA breaks per single strand 66 mega D molecule as a function of treatment time; (bottom) neutral gradients.*

those of V-DNA in that the reduction of the sedimentation rate is dose dependent (Fig. 4) as well as time dependent (Fig. 5), with mostly single-strand breaks and a few double-strand scissions at higher doses. Sodium thiosulfate also inhibited most of the further DNA degradation by chloramine in vitro (Fig. 5).

In addition, single-strand breaks in T-DNA are additive: 640 μM and 1,280 μM chloramine resulted in 1.4 and 3.1 breaks, respectively, per 12 mega D molecule. That the time course study showed 0.6 and 1.4 breaks per 12 mega D molecule in 10- and 30-min treatments also indicates the slow reactivity of chloramine with DNA in vitro.

Endonuclease activity and the sensitivity of DNA to endonucleases after chloramine treatment. Could DNA strand breaks introduced in treated cells result from the potentiation of nuclease activity by chloramine? In fact, treatment of purified *B. subtilis* DNA with cell extract from chloramine-treated cells resulted in fewer breaks in the alkaline-denatured product than with cell extract from untreated cells (Fig. 6).

Is chlorinated DNA sensitized to cleavage by endonuclease? Indeed, we found that after incubating with cell extract, T-DNA is degraded more than control DNA: control DNA has 1.8 new breaks per 12 mega D molecule, whereas

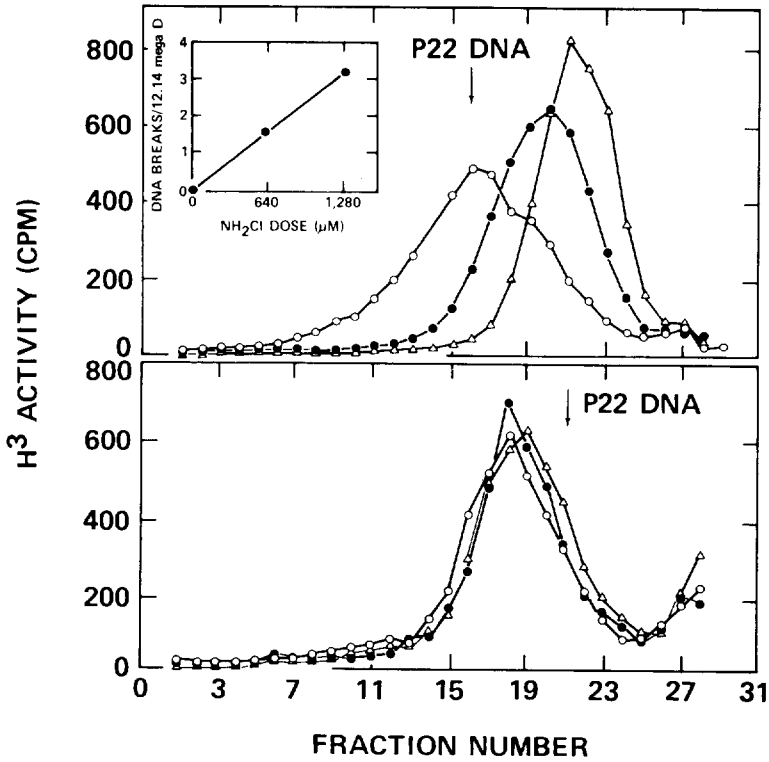

FIG. 4. *Degradation of DNA in vitro with different chloramine doses. SB747 DNA (11 μg/ml), labeled with [^{3}H]thymidine and purified, was treated with different doses of NH_2Cl. About 1 μg of DNA from each treatment was layered on the top of the 5 to 20% linear sucrose gradient and sedimented. P22 [^{3}H]DNA was centrifuged in separate gradients as markers. Average molecular weights for 0- (○), 640- (●), and 1,280- (△) μM chloramine treatments were 12, 4.9, and 2.9 mega D in alkaline gradients and 70, 67, and 60 mega D in neutral gradients, respectively. (top) Alkaline gradients, insert shows DNA breaks per 12 mega D molecule as a function of NH_2Cl doses; (bottom) neutral gradients.*

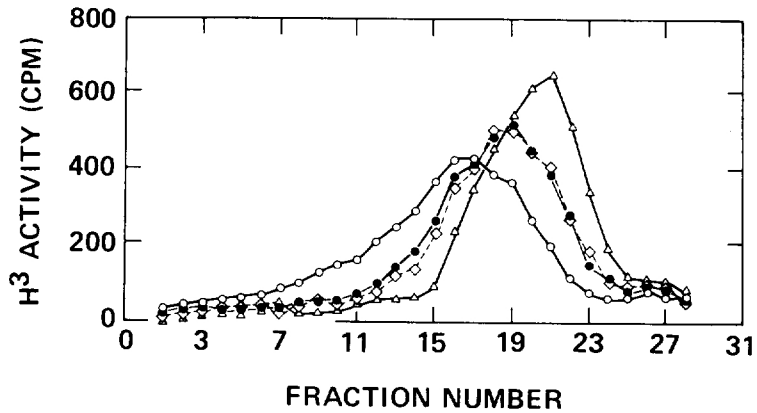

FIG. 5. *Degradation of DNA by chloramine in vitro with different treatment time. SB747 DNA was treated with 640 μM NH_2Cl. Samples were removed at 0, 10, and 30 min and neutralized immediately with equal volumes of 0.1 M sodium thiosulfate. One of the samples removed at 10 min was incubated for another 20 min after the addition of sodium thiosulfate. All the DNA samples were sedimented in alkaline sucrose gradients. The average molecular weights for control (○), 10-min treatment (●), 10-min treatment + sodium thiosulfate + 20-min incubation (◇), and 30-min treatment (△) were 12, 7.5, 7.3, and 4.9 mega D, respectively.*

T-DNA has 1.6 breaks per 7.0 mega D molecule (Fig. 7). During the treatment with endonucleases, both DNA samples were at the same concentration (6.8 μg/ml). Thus, T-DNA sample has 73% more molecules and therefore has 53% more new breaks than that of the control samples. V-DNA exhibits a similar result, with 30% more breaks than its control DNA. These results indicate that chlorinated DNA is sensitized to cleavage by endonucleases.

Alkali-sensitive bonds induced in DNA by chloramine. Do some of the DNA strand breaks of chlorinated DNA observable in alkaline gradients result from lesions (prebreaks) which carry alkali-sensitive bonds? Formamide ($HCONH_2$), a reagent which denatures DNA at

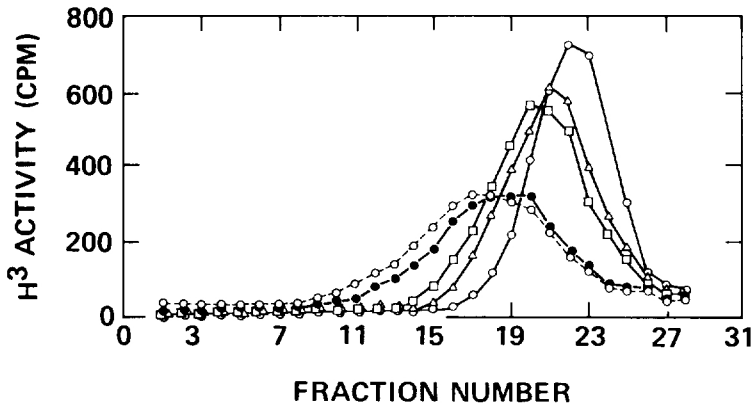

FIG. 6. *Degradation of DNA by cell extract of control and chloramine-treated bacteria. [^{3}H]thymidine-labeled DNA samples (1 μg/ml, equivalent to 3.3 × 10^8 cells/ml) were sedimented in alkaline sucrose gradients after incubating with 168 cell extract (2.5 × 10^8 cells/ml) for 30 min at 37 C. Cell extracts were prepared by incubating cell samples from different NH_2Cl treatments in phosphate buffer-20% glucose-0.3 mg of lysozyme per ml at 37 C. After 40 min of incubation, cell samples were centrifuged and suspended in ice-cold phosphate buffer and kept in ice for 30 min to allow the rupture of the cell membrane before using. Symbols: (– ○–) DNA + cell extract from control with 100% survivor; (△) DNA + cell extract from 32 μM treated cells with 1.3% survivor; (□) DNA + cell extract from 40 μM treated cells with 0.0002% survivor; (●) DNA + cell extract from 56 μM treated cells with 0.00001% survivors; (- -○- -) DNA without treatment.*

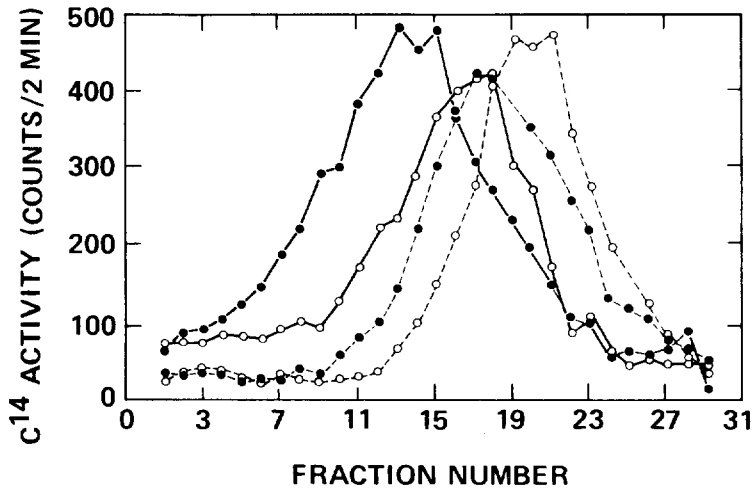

FIG. 7. *Degradation of in vitro chlorinated DNA by endonucleases. SB747 DNA samples treated and untreated with 640 μM NH_2Cl were sedimented in alkaline sucrose gradients after incubating with cell extract from strain 168 for 30 min at 37 C. Cell extract were prepared as described in Fig. 6. Value of 12 mega D for control DNA (– ●–) was used as standard molecular weight. Symbols: (– ○–) chlorinated DNA, 7.0 mega D; (- -●- -) control DNA + cell extract, 4.3 mega D; (- -○- -) chlorinated DNA + cell extract, 2.7 mega D.*

lower temperatures (2), was then used in comparison with NaOH. Control and T-DNA denatured either with alkali or formamide were neutralized and sedimented in neutral gradients. Figure 8 shows that both alkali and formamide treatments expose T-DNA degradation. Whereas formamide-denatured and alkali-denatured control DNA have insignificantly different sedimentation rates (13 versus 12 mega D), formamide-denatured T-DNA is consistently faster sedimenting than that of alkali-denatured T-DNA (10.5 versus 8.5 mega D or 81% versus 71% of the respective controls). V-DNA (extracted from SB566 cells treated with 56 μM chloramine) gave similar results: the formamide-denatured V-DNA and alkali-denatured V-DNA reduced their respective molecular weights to 61 and 48% of their respective controls. DNA whether chlorinated in vitro or in vivo shows a number of alkali-sensi-

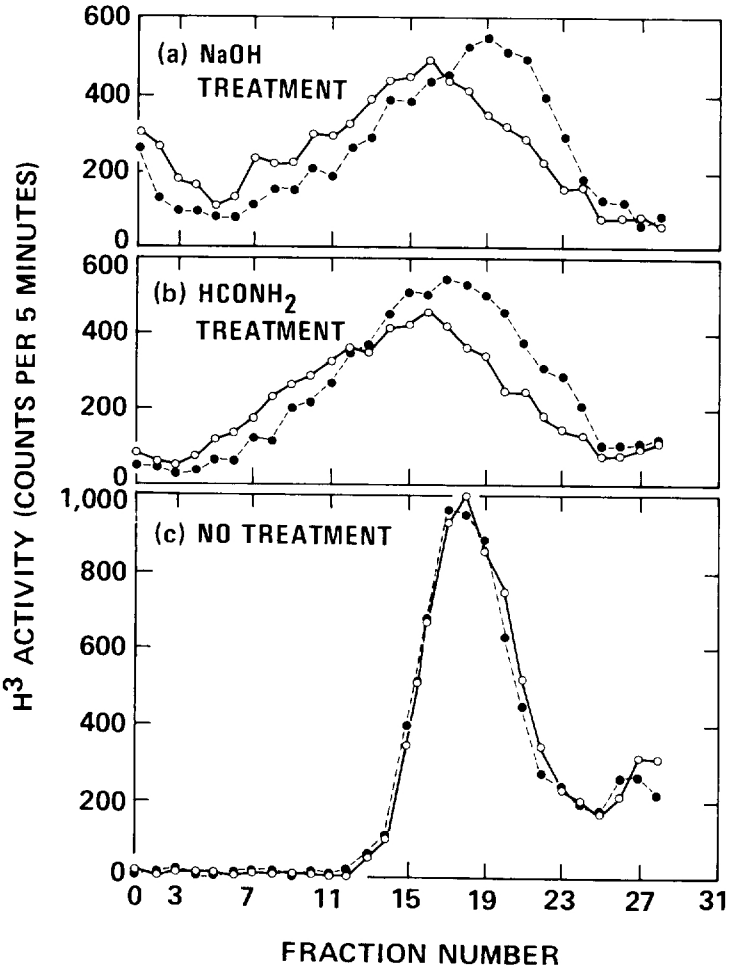

FIG. 8. *Degradation of in vitro chlorinated DNA denatured by sodium hydroxide and formamide. SB747 DNA (55 μg/ml) treated with 3200 μM NH_2Cl was sedimented in neutral sucrose gradients after denaturation. (a) DNA was denatured at a concentration of 27.5 μg/ml in 0.1 M NaOH without added salt. After 5 min at room temperature, the solution was neutralized with one-tenth volume of 1.1 M HCl-0.2 M Tris. (b) DNA was denatured by incubating in 90% formamide at 37 C for 30 min at a concentration 2.75 μg/ml without added salt. (c) DNA was not denatured. All of the samples were then dialyzed twice against 0.05 M phosphate buffer, pH 7.0. Average molecular weight of alkali-denatured control DNA (12 mega D) was used as a marker. The average molecular weight for formamide-denatured control DNA was 13 mega D and the average molecular weights for alkali and formamide denatured-chlorinated DNA were 8.5 and 10.5 mega D, respectively. The native chlorinated DNA does not show a detectable change in average molecular weight compared to the native control DNA. Symbols: (○) control DNA; (●) chlorinated DNA.*

tive prebreaks which are not seen in untreated DNA.

Heat-sensitive sites induced in DNA by chloramine treatment in vitro. The thermal stability of the chlorinated DNA was also tested. Different DNA samples were incubated at 50 C for 5 h and then assayed for transforming activity (16). The results show that V-DNA does not become thermolabile for *tyrA*$^+$-transforming activity (Table 2), whereas T-DNA appears to be thermolabile for single as well as linked markers (Fig. 9). In a separate experiment, further degradation of T-DNA after heating was indeed observed (Fig. 10). The average molecular weight of T-DNA was reduced to 65% whereas the control DNA remained unchanged after heat treatment.

Degradation of DNA of chloramine-treated cells by DNA extraction procedure. Could the extraction procedure degrade V-DNA further? After cells were treated with 56 μM chloramine, both unextracted (as described in Fig. 2) and extracted DNA samples were studied for single-strand breakage. Compared to their respective control, the average molecular weight of single-strand DNA was reduced to 68% without extraction (Fig. 2), whereas the average molecular weight after extraction was reduced to 51% (Fig. 11).

Thus, chlorinated DNA is a labile heterogeneous substance that is sensitized to cleavage by endonucleases and contains single-strand breaks, double-strand scissions, alkali-sensitive sites, thermolabile lesions (T-DNA), and possibly other defects not yet identified by the present study.

DISCUSSION

The reduction in transformation caused by the introduction of defects into *B. subtilis* DNA via chloramine treatment in vivo can be attributed to the failure of the extracted DNA molecule to enter the competent cell as well as the impairment of the process subsequent to entry. By studying the transformation of pneumococcus DNA treated with DNase I, Lerman and Tolmach (9, 10) proposed that the decline in DNA uptake reflects decline in the average molecular weight of the DNA molecules. It is not clear, however, what the correlation between the reduction of DNA uptake and the reduction of the average molecular weight of the DNA molecule is. In chloramine treatment, whereas the average molecular weight of DNA was reduced to 72%, its uptake by the competent cells was only 54%. Other lesions besides DNA strand breaks are possibly involved in the impairment of DNA uptake by distortion or modification of the configuration of the DNA molecules. The difference between DNA uptake and transforming activity of chlorinated DNA also resembles that observed in *B. subtilis*-transforming DNA inactivated with other agents—nitric acid, dimethylsulfate, hydroxylamine, ultraviolet light, etc. (4). It is conceivable that chlorinated DNA, in addition to strand breaks, contains other lesions that do not interfere with DNA uptake but impair the integration or even the eventual expression of the integrated DNA in the competent cells (3).

Heating for 5 h at 50 C further decreased the transformation activity of the T-DNA. The degradation of these heat-sensitive lesions was shown to be a major factor contributing to the further inactivation of the chlorinated DNA. These heat-sensitive lesions, however, allow the unheated DNA to retain its transforming activity.

The seeming heat insensitivity of the DNA extracted from chloramine-treated cells, as compared to in vitro-treated DNA, could be explained in different ways. As the process of DNA extraction does induce more breaks in V-DNA, the thermolabile sites could be broken during extraction procedure. This speculation, however, is not yet proven. Other possibilities could be that the heat-sensitive lesions were never introduced in vivo, or that the lesions induced could be either immediately repaired or cleaved in vivo.

DNA degradation continues only slowly in chlorinated cells after sodium thiosulfate is added. The direct reaction of chloramine with DNA is the most plausible explanation for the DNA breaks induced in vivo. However, we cannot exclude a dynamic equilibrium of endonucleolytic breaks and enzymatic repair, the latter being the target of the chloramine. We have

TABLE 2. *Effects of heat treatment on DNA from chloramine-treated cells*[a]

NH_2Cl (μM)	*tyrA*$^+$-transforming activity (%)	
	Not heated	Heated
0	100[b]	95.76
56	22.32	22.93
80	18.94	21.25

[a] DNA was extracted from SB566 cells after chloramine treatment in vivo. DNA samples were divided into two parts; one part was kept at 5 C and the other part was incubated in a 50 C water bath for 5 h, and then transformation was performed. DNA concentration was 0.42 μg/ml during transformation.

[b] The absolute number was designated as 100%. The other results were compared to this.

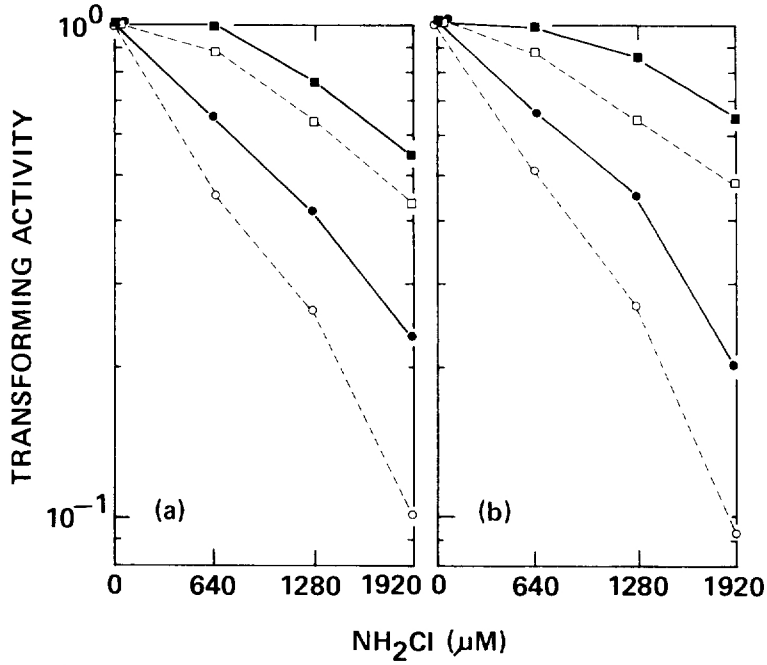

FIG. 9. *Effects of chloramine treatment and heat treatment in vitro on DNA-transforming activity. Transforming DNA of strain 168 (trpC) was treated with different doses of NH_2Cl at a concentration of 2.9 μg/ml and then DNA samples were dialyzed twice against 0.05 M phosphate buffer after the addition of sodium thiosulfate. Heat treatment and transformation were performed as described in Table 2. DNA concentration was less than 0.145 μg/ml during transformation. Cotransfer efficiency of each treatment was determined by replicating 200 $hisB^+$ or $tyrA^+$ transformants onto phenylalanine-, histidine-, and tyrosine-deficient media. The single marker activity and the linked marker activity of unheated control DNA were used as the standard and were designated as 10°. Symbols: (a) (●) $hisB^+$; (○) $hisB^+$ after heating; (■) ($aroB^+$ $hisB^+$ $tyrA^+$)/$hisB^+$; (□) ($aroB^+$ $hisB^+$ $tyrA^+$)/$hisB^+$ after heating. (b) (●) $tyrA^+$; (○) $tyrA^+$ after heating; (■) ($aroB^+$ $hisB^+$ $tyrA^+$)/$tyrA^+$; (□) ($aroB^+$ $hisB^+$ $tyrA^+$)/$tyrA^+$ after heating.*

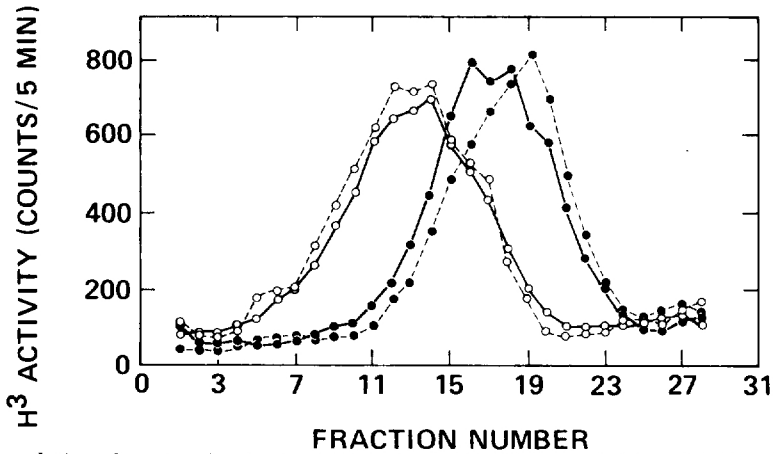

FIG. 10. *Degradation of in vitro chlorinated DNA by heat. [^{3}H]DNA purified from SB747 cells was treated with 640 μM NH_2Cl at a concentration of 2.7 μg/ml. The control and chlorinated DNA samples (1 ml) were dialyzed twice against 1 liter of 0.05 M phosphate buffer, pH 7.0, after the addition of sodium thiosulfate. After dialyzing, heat treatment was performed to a part of each sample as described in Table 2. Transformation and sedimentation in alkaline sucrose gradients were then performed. DNA concentration was less than 0.27 μg/ml during transformation. Symbols: (—○—) control DNA, 12 mega D, 100% $tyrA^+$-transforming activity; (- -○- -) heated control DNA, 12 mega D, 99% $tyrA^+$-transforming activity; (—●—) chlorinated DNA, 5.0 mega D, 62% $tyrA^+$-transforming activity; (- -●- -) heated chlorinated DNA, 4.1 mega D, 34% $tyrA^+$-transforming activity.*

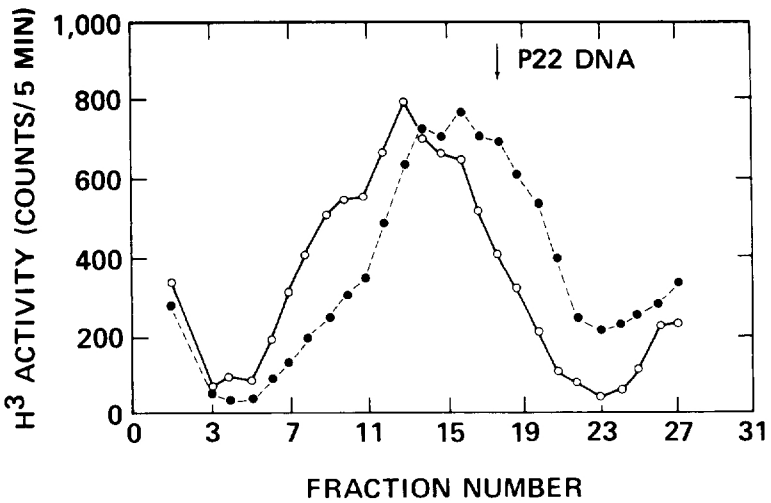

FIG. 11. *Degradation of DNA by DNA extraction procedure. DNA samples extracted from NH_2Cl-treated (56 μM) and control SB566 cells were prepared as described Materials and Methods for transforming DNA and were then sedimented in alkaline sucrose gradients. The average molecular weight of control DNA (○) was 41 mega D and chlorinated DNA, 21 mega D (●).*

already noted that chlorinated DNA is sensitized to further cleavage by endonucleases, which recalls similar behavior of alkylated DNA (17). Although DNA was not vigorously extracted in studying the strand breaks of DNA chlorinated in vivo, some breaks could still be induced by the procedure of making cell lysates. Bearing these factors in mind, the efficiency of the reaction of chloramine with DNA may be compared between in vitro and in vivo treatments. The chloramine effect in vivo is nearly additive from 56 to 112 μM, therefore 56 μM (112 μM − 56 μM) chloramine can be considered to produce 3.1 (3.6 − 0.5) breaks per 65 mega D molecule. The calculation is shown in Table 3. Averaging the results from other experiments by treating cells from the same culture (4.1×10^8, 4.7×10^8, and 3.0×10^8 breaks/1 ml of 1 μM chloramine), 1 ml of 1 μM chloramine can induce 4.3×10^8 breaks for 30 min in vivo treatment. Chloramine (1,280 μM) induced 3.1 breaks per 12 mega D in vitro (Table 3). Averaging the results with other three experiments (breaks per 1 ml of 1 μM chloromine 1.4, 1.4, and 1.2×10^9), 1 ml of 1 μM chloramine induces 1.3×10^9 DNA single-strand breaks. Thus, chloramine is about three times more efficient in vitro than in vivo in reacting with DNA to produce breaks.

Studies to correlate the mutagenic efficiency of chloramine to the present findings are reported in a second paper.

TABLE 3. *DNA breaks induced in vivo and in vitro by chloramine treatment*

Chloramine treatment calculations		
Determinants	In vivo $B = ([m_1/m_2]b\ c_1)/d$	In vitro $B = ((m_1/m_2)b[c_2/w])/d$
Mol wt of *B. subtilis* DNA (m_1) (8)	2×10^9 daltons	2×10^9 daltons
Avg mol wt of control DNA molecule (m_2)	6.5×10^6 daltons	12×10^6 daltons
Breaks per control of DNA molecule (b)	3.1	3.1
Concn of cells during treatment (c_1)	3.14×10^8/ml	
Concn of DNA during treatment (c_2)		11 μg/ml
Wt of *B. subtilis* DNA (w)		3.33×10^9 μg
Chloramine dose (d)	56 μM	1,280 μM
Breaks/1 ml of 1 μM chloromine (B)	5.4×10^8	1.3×10^9

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