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Effects of Thermoradiation Treatments on the DNA of *Bacillus subtilis* Endospores

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Abstract

Endospores of the bacterium, *Bacillus subtilis*, have been shown to exhibit a synergistic rate of cell death when treated with particular levels of heat and ionizing radiation in combination. This synergism has been documented for a number of different organisms at various temperatures and radiation doses (Sivinski, H.D., D.M. Garst, M.C. Reynolds, C.A. Trauth, Jr., R.E. Trujillo, and W.J. Whitfield, "The Synergistic Inactivation of Biological Systems by Thermoradiation," Industrial Sterilization, International Symposium, Amsterdam, 1972, Duke University Press, Durham, NC, pp. 305-335). However, the mechanism of the synergistic action is unknown. This study attempted to determine whether the mechanism of synergism was specifically connected to the DNA strand breakage--either single strand breakage or double strand breakage. Some work was also done to examine the effect of free radicals and ions created in the spore body by the radiation treatments, as well as to determine the functionality of repair enzymes following heat, radiation, and thermoradiation treatments. *Bacillus subtilis* spores were treated at combinations of 33 kr/hr, 15 kr/hr, 105° C, 85° C, 63° C, and 50° C. Some synergistic correlation was found with the number of double strand breaks, and a strong correlation was found with the number of single strand breaks. In cases displaying synergism of spore killing, single strand breakage while the DNA was in a denatured state is suspected as a likely mechanism. DNA was damaged more by irradiation in the naked state than when encased within the spore, indicating that the spore encasement provides an overall protective effect from radiation damage in spite of free radicals and ions which may be created from molecules other than the DNA molecule within the spore body. Repair enzymes were found to be functional following treatments by radiation only, heat only, and thermoradiation.

Acknowledgements

The authors thank Drs. Barbara and Peter Setlow for their invaluable assistance in outlining various microbiological procedures specific to this work, and Dr. Wayne Nicholson and Tony Slieman for their equally critical laboratory assistance in the subtleties of several procedures.

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I. Introduction

Previous research shows evidence of a synergism between heat and radiation on the killing of bacterial spores (Sivinski, 1971, 1972). The mechanism for this synergism is unknown. Some understanding of the mechanisms responsible for cell death from radiation or heat individually does exist, but this information is far from conclusive. The purpose of this research was to search for evidence indicating whether deoxyribonucleic acid (DNA) strand breaks, either double or single, might be the primary mechanism of synergistic cell death by thermoradiation. This knowledge would enhance our understanding of the working of the bacterial spore, an important and somewhat poorly understood biological structure in the fields of microbiology and bioterrorism.

II. Background

Previous research shows evidence of a synergism between heat and radiation on the killing of bacterial spores (Sivinski, 1971, 1972). The mechanism for this synergism is unknown. Most of the detailed research on thermoradiation of *Bacillus subtilis* was done by the Planetary Quarantine Commission at Sandia National Laboratories in the early 1970's. This organization was tasked with examining ways to sterilize the sensitive equipment on interplanetary spacecraft so that the spacecraft could return with samples from other planets that had not been contaminated by the spacecraft itself. The advantage of the synergy of thermoradiation was that it could allow lower levels of each energy--radiation and heat--to be used. This could sterilize equipment that would otherwise have been damaged by the high levels required of radiation or heat alone. Figure 1 shows the results published by the PQC for radiation at 27 kilorad/hour (kr/hr), and heat at 105° C.

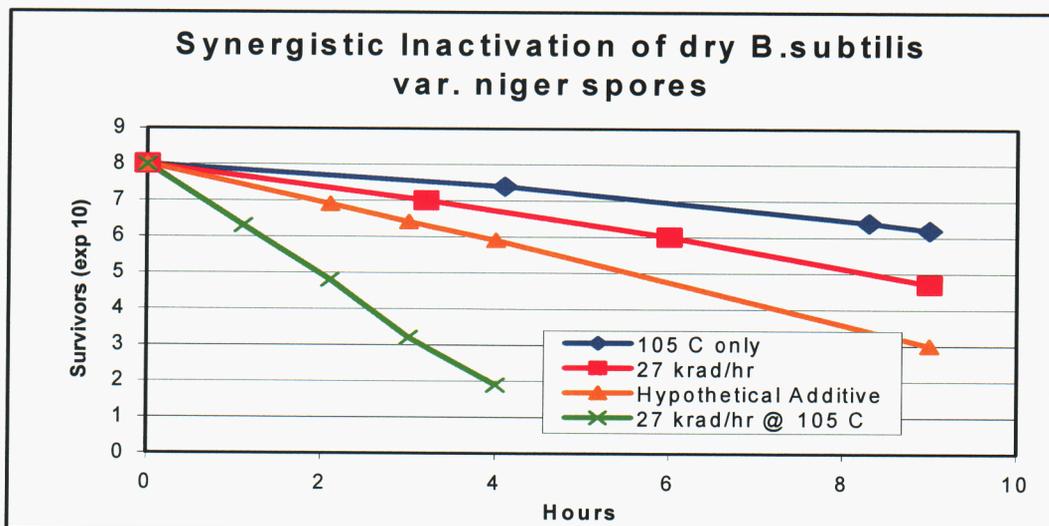


Figure 1. Results of PQC study on *Bacillus. subtilis* var. niger (Sivinski, 1972).

The PQC also did some studies on organisms other than *B. subtilis*. They did a cursory study on *Bacillus pumilus*, another spore-forming bacteria, as well as the enzyme lysozyme, the bacteriophage T4, and a non-spore-forming Gram-negative bacteria, *Escherichia coli B*. The primary functions of each of these biological agents were found to be subject to the thermoradiation effect, although the relevant temperature and radiation ranges varied for each agent. For *B. pumilus* and *E. coli B*, the measured function was the ability to reproduce, as it was in *B. subtilis*. For lysozyme, the evaluated function was its ability to lyse a suspension of *M. lysodeikticus* cell walls. For the bacteriophage T4, the evaluated function was its ability to lyse its host organism, *E. coli B* (Devore, 1987).

Other researchers have also found a synergistic effect from thermoradiation on various organisms. Fisher and Pflug carried out experiments with *B. subtilis* var. niger similar to those of the PQC and found similar results (Fisher, 1977). They also determined that dry heat is more effective in the thermoradiation synergistic effect than is wet heat.

The DNA molecule is widely considered to be the primary target for spore killing by ionizing radiation. DNA is made up of two separate strands of polymers, each consisting of alternating sugar and phosphate molecules. These alternating sugar and phosphate molecules are frequently called the backbone of the DNA strand. The bases of DNA are bonded to the sugar molecules. Bases on each of the two separate strands form hydrogen bonds with the bases on the opposite strand—this arrangement holds the DNA in its well-known two stranded helical form. Figure 2 illustrates this structure of DNA.

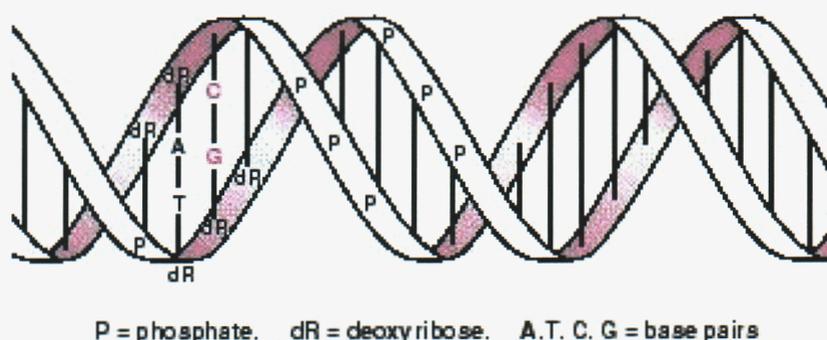


Figure 2. Two stranded helical structure of DNA.

Bases between strands always order themselves such that a purine base is opposite, and hydrogen bonded to, a pyrimidine base. The purine bases are adenine and guanine, which are found opposite, respectively, thymine and

cytosine, for *B. subtilis*. Hence bonds within the DNA molecule could be broken between strands (the hydrogen bonds between base pairs) or within strands (the bonds connecting the sugar and phosphate molecules). The breakage within strands can be either a single strand break (ssb), meaning that only one side of the double helix has a break in its sugar-phosphate backbone, or a double strand break. A double strand break (dsb) is generally described as breaks in both DNA strands within about 10 base pairs of each other (Holley, 1996). Enzymes within the cell repair strand breaks, and presumably single strand breaks should be much easier to repair than double strand breaks. The effect of single strand breaks is not conclusively known, but it is the double strand break which has received most of the blame for cell death from ionizing radiation over the past twenty to thirty years of study (Goodhead, 1994). However, there is conflicting experimental evidence on this subject. According to Goodhead, the correlation between the initial number of double strand breaks and radiosensitivity is generally not good. Goodhead argues that not all dsb are identical either in type or effect, and so the division of strand breaks into only double or single strand breaks is too simplistic. Presumably, then, the location of the strand break and the existence, or lack of, others nearby is critical. Such a clustering of damage has been examined in mammalian cells and found to increase the biological effectiveness of the ionizing radiation (Goodhead, 1994). However, Setlow (1992) states unequivocally that UV and gamma radiation kill spores through DNA damage. The effects of UV radiation may be significantly different from those of ionizing radiation. Setlow reports that the bacterial endospore state is less susceptible to gamma radiation than the vegetative state, as might be expected. However, this difference in sensitivity for gamma radiation is less than for UV radiation or for heat (P. Setlow, 1992). In addition, small acid-soluble proteins present in the endospore, which provide protection from UV radiation, do not appear to have any protective abilities against ionizing radiation (P. Setlow, 1992). Radiation appears to damage the cell membrane of eukaryotic cells, which may contribute to cell death through the oxidation of sulfhydryl groups of the membrane-binding bound proteins (Kim, 1996). Another theory holds that certain damage to the cell membrane may initiate apoptosis, a programmed cell death in eukaryotic cells (Cohen-Jonathon, 1999).

More likely though, is the proposal of DNA as the primary radiation target. An experiment using mammalian cells showed that the yield of double strand breaks was linear with the radiation dose (Ward, 1991). Because these cells are eukaryotic, rather than prokaryotic, the effects of radiation on them may be quite different from the bacterial spores used in this study. Work with dilute solutions of DNA (not encased within a cell) have produced conflicting arguments regarding the possible existence of a dose squared relationship with the number of double strand breaks (Chatterjee, 1985, Hutchinson, 1985). Hutchinson gives a theoretical argument that the number of double strand breaks is proportional to the square of the dose of ionizing radiation, a result of the coincidence of separately formed single strand breaks, thus effectively

creating a double strand break. However, Chatterjee claims that the probability of this occurring is almost zero ($\sim < 10^{-6}$).

Effects of radiation on a cell can be divided into two categories, direct and indirect. Direct effects consist of x-ray energy that is deposited directly into the target, generally assumed to be the DNA molecule, breaking a chemical bond in the DNA molecule. The direct effect would appear to be simply a question of probability: as the radiation particle deposits energy within the cell, energy deposition occurs within the DNA molecule in proportion to the volume of the DNA molecule within the cell. In vegetative cells this is a relatively small proportion; in spores it is much higher. The bacterial spore has dispensed with any sections of the cell that are not necessary for its hibernation, leaving essentially the spore coat, the DNA, and a small amount of water. This water is the subject of great discussion regarding indirect effects.

Indirect effects occur when the radiation particle's energy is deposited in water molecules throughout the cell. In this situation, the bonds in the water molecule are then broken, producing free radicals and ions. This radiolysis of H_2O yields H_2 , H_2O_2 , H^+ , OH^- , with intermediate products, $\bullet H$ and $\bullet OH$ (Choppin, 1980). These products then react, yielding H_2 , H_2O_2 , $\bullet H_{aq}$, $\bullet OH_{aq}$, e^-_{aq} , OH^- , and H_3O^+ . Aerated solutions may also have $HO_2\bullet$, H_2^+ , and O^- . Of these radiolysis products, the hydroxyl radical ($\bullet OH$) is assumed to have the most devastating effects on the cell's DNA (Henle, 1995). A theoretical calculation of the average hydroxyl radical's migration distance through a cell is 3.5 nm (Chatterjee, 1992). Of the $\bullet OH$ entities reaching and reacting with the DNA molecule, it is believed that approximately 20% would react with the sugars, while the remaining 80% react with the bases (Chatterjee, 1992). Based on these reaction percentages, some researchers have proposed that base damage does not lead to strand breaks, only sugar damage does. If base damage were a cause of strand breaks, they argue, then the surviving fractions would be much lower than they have been reported to be (Chatterjee, 1990). No experimental data has as yet proven or disproven this assumption. Of course, this theory also incorporates the assumption that strand breaks are the cause of cell death. In addition to single and double strand breaks, the interaction of $\bullet OH$ with DNA may lead to protein-DNA cross-linkage, or other base alterations that could be fatal. Unfortunately, it is very difficult to ascertain the actual amount of water contained in an endospore, and therefore no clear data on this subject is available (P. Setlow, 1995). It is estimated that the spore core has 28-57% wet weight water content, as compared to approximately 80% in a growing cell (P. Setlow, 1992); however, in the spore this is not free water, but is water that is complexed to other molecules within the spore. The configuration of the water will, of course, affect its production of free radicals and ions.

The spore has a number of damage control techniques, as well as the ability to repair some DNA strand breaks. The sole purpose of the endospore is to protect the cell from unfavorable environmental conditions, so it is well suited for

this task. As discussed above, the spore has a lowered water content, decreasing the hazards from radiolysis. The spore core is surrounded by a thick protein layer known as the spore coat. This, along with a peptidoglycan cortex and a membrane around the spore coat, restrict access to the spore core. However, it is unclear to what extent these environmental protectors play a role in limiting damage from ionizing radiation. During the formation of the spore, the DNA in the forespore (early spore) becomes saturated with a type of proteins known as small, acid-soluble proteins (SASP's) of the α/β type (P. Setlow, 1995). These proteins seem to play a significant role in protecting the spore's DNA, particularly from ultraviolet radiation. A number of studies have shown that spores lacking these particular proteins are much more sensitive to UV radiation, heat, hydrogen peroxide, and desiccation than those with the proteins. However, as mentioned earlier, these proteins do not appear to convey protection from ionizing radiation (B. Setlow, 1994, 1995, 1996, May 1998, P. Setlow, 1992, 1995).

The effects of heat on bacterial spores is not conclusively well known. There are a number of possible targets within the spore. Some possible mechanisms by which heat may inactivate a cell are 1) damage of DNA; 2) inhibition of protein synthesis; 3) damage of the cell membrane; and 4) inactivation of critical metabolic enzymes (Kim, 1996). Damage to the DNA may be through depurination (P. Setlow, 1992), although because of the protection of α/β type SASP's DNA damage is not believed to be a significant contributor to killing of spores by heat (B. Setlow, Oct 1998). Some studies have shown that treatment with wet heat, while more effective at killing spores, does not produce as many DNA mutations as does treatment with dry heat (Fairhead, 1993, P. Setlow, 1995). Protein denaturation and enzyme inactivation are also possible culprits in heat killing of spores (B. Setlow, Oct 1998), although there is little conclusive evidence concerning this mechanism.

Given the myriad possibilities for the mechanisms of radiation and heat damage individually to spores, clearly the mechanism for damage from simultaneous radiation and heat energies has many possibilities as well. Previous studies have not attempted to determine what this mechanism might be, but have provided some information about the synergistic effect on the survival rate of spores treated with thermoradiation. In addition, some theories of the mechanism responsible have been proposed. Many types of biological entities have exhibited synergistic inactivation from thermoradiation: proteins, viruses, spores, bacteria, yeast, mammalian cells, and human cancers (Dugan, 1971). The studies by the Planetary Quarantine Commission at Sandia National Laboratories in the early 1970's found that only within a critical temperature and dose range is a temperature dependent radiosensitivity observed for any given biological system (Dugan, 1971).

III. Method

The endospore-forming bacteria *Bacillus subtilis* was selected for this study due to its frequent use in previous studies of thermoradiation synergy. The particular strain, PS533, was the generous gift of Barbara and Peter Setlow of the University of Connecticut. This bacteria has a 4500 base pair plasmid, which is normally in a supercoiled circular form. A bacteria with such a plasmid was chosen because individual strand breaks in this supercoiled plasmid can be more easily detected than individual breaks in the genomic DNA molecule, and as such is useful as a modeling system (Swenberg, 1995). The strand break yields of plasmid DNA as compared to genomic DNA have been found to be proportional in *Escherichia coli*, another bacterium (Hutchinson, 1985). The genomic DNA for *B. subtilis* is approximately 4300 kilo-base pairs in size. Therefore, each plasmid represents a target of about 0.1% the size of the genomic DNA molecule. However, there are, on average, 10 copies of this plasmid in each bacterial cell, so the target of total plasmid DNA (pDNA) is roughly 1% the size of the target of genomic DNA (gDNA). In order to see damage to the pDNA, then, it will be necessary to cause an amount of damage that would result in about 100 strand breaks in the gDNA in a cell. For this reason, extensive treatment with both radiation and heat were required for this experiment.

A. Preparation of spores

B. subtilis spores were grown in 2xSG broth, consisting of, per liter:

16.0 g Difco brand nutrient broth
2.0 g KCl
0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.

The broth was placed in a container large enough that the solution only filled 15-20% of the volume, in order to allow proper aeration of the growing cells. The pH was adjusted to 7.0, then the solution was autoclaved for sterilization.

The following sterile component solutions were added to one liter of cooled (55°C) medium:

1.0 mL 1 M $\text{Ca}(\text{NO}_3)_2$
1.0 mL 0.1 M $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
1.0 mL 1mM FeSO_4
2.0 mL 50% (weight/volume) glucose.

The solution was then inoculated with spores from a previously grown LB agar plate using a sterile wire loop. The spores were incubated at 37° C with vigorous shaking for 5 days.

Upon removal from the incubator, the spores were sonicated at high level for 2 hours, then centrifuged at 20,000 g for 10 minutes. The supernatant was removed, and the pellet resuspended in ¼ of the original volume of cold distilled water. This sequence of centrifugation and resuspension was repeated two more times, then the suspension was shaken overnight at 4° C. The spores were examined in a phase contrast microscope for purity. If vegetative cells remained present at greater than 5%, then additional centrifugation (10,000 g, 20 min), resuspension, and shaking was employed until the pellet achieved a spore concentration level of 95%. When the desired purity was reached, the suspension was centrifuged a final time, the supernatant removed, and the minimum amount of cold distilled water necessary to fully and uniformly resuspend the pellet was added.

The optical density of this suspension at 600 nm was measured. Using this measurement, the volume of the suspension that was required to obtain 50 OD600 units, approximately equal to 5×10^9 spores, was determined. 50 OD600 units of *B. subtilis* spores were placed on each microscope slide for treatment, in a spot size of about one centimeter in diameter. The placement of the spore spot on each slide was predetermined by the dosimetry analysis from the x-ray beam.

Spores were allowed to dry completely on the slides by placing them in a negative airflow ventilation hood for 60-90 minutes. Once dry, the spores were covered by slide covers taped securely in place over each spot.

The slides were then placed in cardboard slide carriers and transported under ambient conditions to Sandia National Laboratories for treatment, a distance of roughly 10 miles. When not being treated or otherwise handled, spores on slides were stored in a freezer at -5° C.

B. Treatment of spores

Spores were irradiated at Sandia National Laboratories using a Philips 225 kVp x-ray machine. They were also heated, using a standard laboratory hot plate. The following matrix shows the various combinations of heat and radiation treatments that spores samples received:

Table 1: Matrix of radiation and heat treatments.

	R ₀ T ₁	R ₀ T ₂	R ₀ T ₃	R ₀ T ₄	R ₁ T ₀	R ₁ T ₁	R ₁ T ₂	R ₁ T ₃	R ₁ T ₄	R ₂ T ₀	R ₂ T ₁	R ₂ T ₂	R ₂ T ₃	R ₂ T ₄
1 hr	√	√	√	√	√	√	√	√	√	√	√	√	√	√
8 hrs	√	√	√	√	√	√	√	√	√	√	√	√	√	√
12 hrs	√	√	√	√	√	√	√	√	√	√	√	√	√	√
16 hrs	√	√	√	√	√	√	√	√	√	√	√	√	√	√

The notations R_0 , R_1 , and R_2 represent radiation levels from the Philips x-ray machine of 0 krad/hr, 33 krad/hr, and 15 krad/hr. Similarly, the notations T_0 , T_1 , T_2 , T_3 , and T_4 represent settings of the hot plate; T_0 indicates that the hot plate was not turned on, at T_1 the temperature was 105° C, at T_2 the temperature was 85° C, at T_3 the temperature was 63° C, and at T_4 the temperature was 50° C. Note that T_0 does not represent 0° C, but laboratory room temperature, approximately 23° C.

The experimental setup under the x-ray beam was as shown in Figure 3.

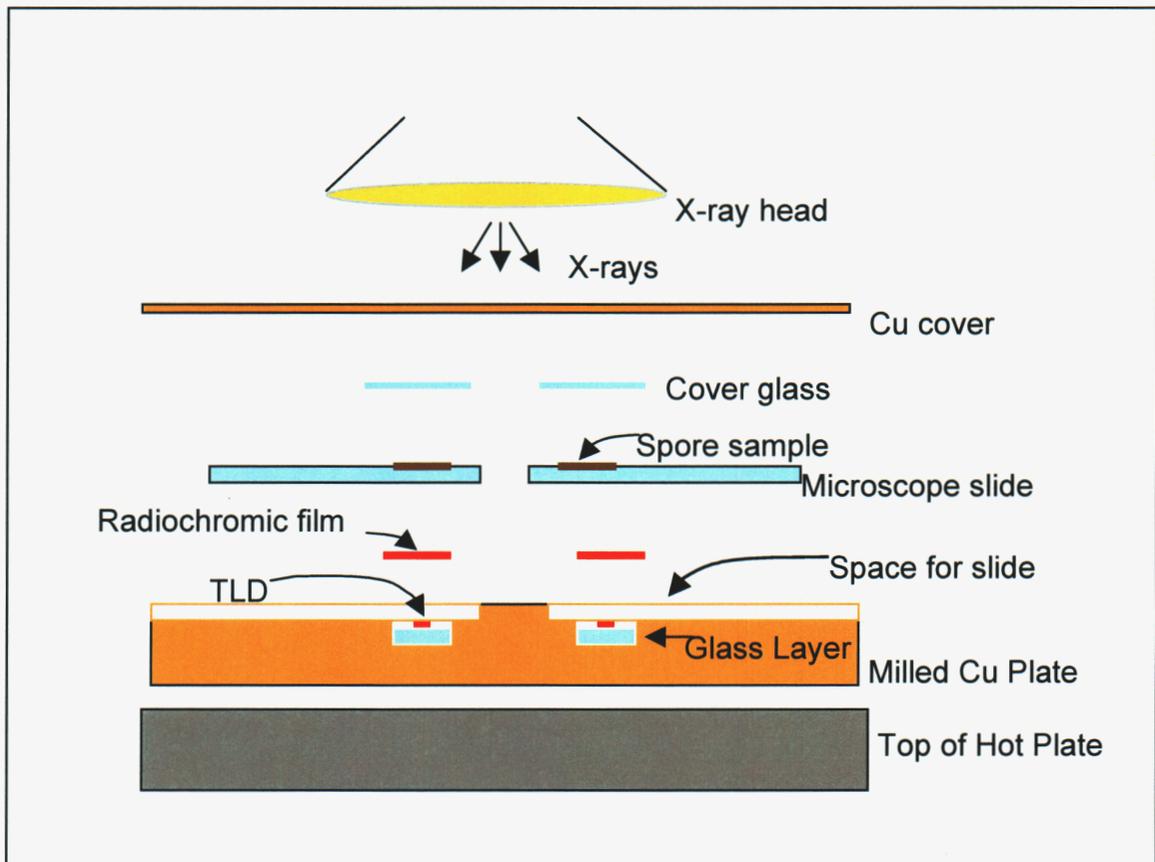


Figure 3. Experimental setup.

To improve heating consistency, a copper plate was sized to fit the top of the hot plate, and machined to hold 4 microscope slides precisely. A thin sheet of copper was sized to act as a cover. In order to determine locations of uniform dose rate, the x-ray beam was characterized. Several types of dosimeters were used. First, to get an estimate of the whole beam map, full-sheet Gafchromic radiosensitive film was centered under the beam. Figure 4 shows the result of this beam mapping. Color changes show the changes in dose.

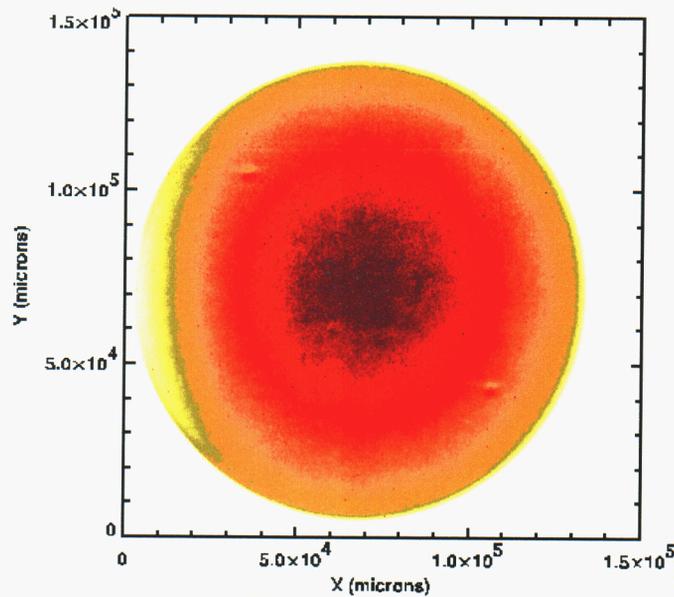


Figure 4. Beam map of Philips 225 kV x-ray machine.

Based on this full-beam map, several likely isodose locations were evaluated with 1 cm^2 pieces of Gafchromic film. These 1 cm^2 sections were placed in a Far West Technologies reader to produce specific values of dose received, enabling determination of four 1 cm^2 locations of identical dose rate. These four positions were then used as locations for the 1 cm diameter spore spots on the microscope slides. The radiosensitive film was used to continually verify the dose rates received by the spores throughout the experiment.

Because this radiosensitive film is not usable at high temperatures, small squares were milled out in the copper plate beneath the locations of the microscope slides in order to hold thermoluminescent dosimeter (TLD) chips. The TLD chips are not affected by the temperatures used in this experiment and so could measure dose rate during heat and irradiation treatments.

Dose readings were initially taken with the radiosensitive film in the place of the spore spot on the microscope slide. A thin glass slide cover was in place over the film. The film itself was encased in a thin foil pouch for protection from light

radiation. The picture in Figure 5 shows the slide and copper plate arrangement. The glass slide cover is 0.15 mm thick, allowing for equilibration of the radiation and a consistent dose to the radiosensitive film or spore spot located beneath.



Figure 5. Copper plate milled for holding microscope slides.

Heating of spore samples was achieved by a standard laboratory hot plate. As discussed above, to achieve greater heating consistency, a 3/8" copper plate was used to hold the microscope slides. During heat treatments, this copper plate was heated to temperature, with a thin copper cover in place. Temperature of the plate was measured by inserting a narrow wire thermocouple into holes drilled into the edges of the copper plate. These holes reached 3" into the copper plate, far enough to measure the plate temperature directly beneath the designated locations for the spore samples. When the desired temperature was reached, the thin copper cover was removed briefly and the microscope slides containing the spore samples were placed in their milled slots within the thick copper plate, then the heated copper cover was replaced. Monitoring the thermocouple readings following this procedure revealed that the temperature within the copper plate recovered to within 1% of the original, desired temperature within 10 minutes. The temperature did not

drop more than 7% from the initial placement of the room temperature microscope slides into the heated copper plate. If spores were to be irradiated and heated at the same time, the x-ray machine was turned on within one minute of placement of the microscope slides on the heated copper plate.

C. Evaluation of treated spores

Extraction of DNA from spores is a difficult process. The procedure for doing so was generously detailed by Barbara Setlow, with additional critical assistance provided by Wayne Nicholson and Tony Slieman of the University of Arizona.

Spores were removed from the laboratory slides by covering them with a thin layer of 10% (weight/volume) polyvinyl alcohol (PVA), ensuring that bubbles did not interfere with the liquid contacting all spores to be removed. Slides were placed in a negative air flow ventilation hood for approximately 90 minutes, or until the PVA was just dried. PVA solidifies into a flexible plastic, but if overdried becomes very difficult to remove. Once dried, the PVA was removed using a sterilized razor blade and tweezers.

The PVA/spore spot was placed into a 1.5 mL microcentrifuge tube containing either sterilized water (for development of a survival rate) or decoating solution (for extraction of DNA). The decoating solution consists of, for 1 mL:

15 mg DDT
100 μ L NaCl
50 μ L 10% SDS
10 μ L 10 M NaOH
840 μ L distilled H₂O.

Spores were thoroughly resuspended in this solution, then heated at 65° C for 30 minutes. Spore coats must be removed before the spores will be susceptible to lysozyme. Spores were then centrifuged and washed 5 times with 1 mL of the solution: 10 mM Tris, pH 7.5; 10 mM EDTA; 150 mM NaCl. Next, spores were pelleted and suspended in 1 mL of buffer "B1." Suspension in this buffer is the first step in a commercially produced DNA removal kit from Qiagen, the Genomic-tip kit. The procedures and chemical composition of buffers of this kit are contained in Appendix A.

For neutral gel analysis, a 1% agarose gel was prepared by mixing 0.5 g DNA agarose with 50 mL of 1xTAE; mixture was microwaved until boiling, approximately one minute at 50% power. 100 μ L of 2 mg/mL ethidium bromide was added, then the gel was poured into a gel tray and the well comb added. DNA to be loaded was placed on plastic stretch wrap, mixed by pipeting up and down with 2 μ L of the standard "Blue Juice" loading buffer, and 7 μ L loaded into each DNA well. Two microliters of DNA mass marker was also loaded into one well on each gel. The gel was run at 100 V for approximately 90 minutes in

1xTAE. The DNA molecule is negatively charged; therefore by placing charges at each end of the gel, the DNA will migrate toward the positive charge. The resistance provided by the gel allows smaller segments of DNA to move more quickly towards the positive charge, thus separating differently sized segments by distance traveled through the gel. Because ethidium bromide attaches itself to the DNA molecule and fluoresces under UV light, this procedure allows one to see different sizes of DNA segments in the gel by using a UV light table.

For alkaline gel analysis, the gel was made with deionized water instead of 1xTAE, and the ethidium bromide was left out of the gel, as this interferes with the alkalinity. Before the gel was poured, but after it cooled to $<60^{\circ}$ C, the following was added:

250 μ L 10 M NaOH
500 μ L 0.1 M EDTA (pH= 8.0).

Again, in order to preserve the alkaline nature of the gel, a different loading buffer was used, consisting of: 300 mM NaOH, 6 mM EDTA, 0.15% bromocresol green. This gel was run at 70 V for about 100 minutes in alkaline soaking buffer, consisting of:

1.25 mL 10 M NaOH
2.5 mL 0.1M EDTA
246 mL deionized water

This gel becomes very hot, so the entire setup was placed in ice for the duration of the electrolysis.

Following the alkaline gel electrolysis, the gel was removed from the gel tray and placed in 100 mL of neutralizing solution, floating freely:

12.1 g Tris base
1.7 g NaCl
deionized water to make 100 mL, pH=7.6

This combination was placed on a gentle shaker for 30-45 minutes.

The gel was then removed from the neutralizing solution, rinsed with deionized water, then placed in rinsing solution of

100 mL 1xTAE
200 μ L ethidium bromide.

and put on gentle shaker for 30-45 minutes.

The gel was destained by removing it from the rinsing solution and placing it in 100 mL 1xTAE on a gentle shaker for 30-45 minutes. The gel was then analyzed on UV light table.

The gels produced by this electrophoresis procedure can be analyzed qualitatively by visual inspection; however for the purposes of this research, more quantification was needed. Therefore, a software program, TotalLab, a product of the software company Phoretix, was used. This program is designed to analyze and quantify the results of gel electrophoresis. All of the data values presented in this report as amounts and sizes of DNA present, were obtained using this program. Representative gel pictures can be seen in Appendix B.

The survival curve is generated by culturing cells that have been exposed to the treatments of interest. Treated spores were removed from the laboratory slides as described above using PVA. These spores were then resuspended in sterilized water. Serial dilutions were then performed using this suspension, and 100 μ L samples from various dilutions were cultured on agar plates. By counting the number of colonies grown on the plate, then multiplying by the inverse of the dilution, one can determine the number of colony forming units (assumed to be equal to viable spores) that were originally present.

In addition to the treatments on spores described in this section, an additional treatment was done on naked DNA—DNA was removed from its cell, then dried on a laboratory slide, irradiated, and analyzed for strand breaks as described above.

A representative sample of spores was tested for enzyme activity. Spore spots were removed as usual from the microscope slides, but were then resuspended in 2xSG broth and placed in an incubator, with shaking, for 2 hours at 37° C. These spores were then removed from the incubator and examined for DNA breakage and survival as usual.

IV. Data Results

Figures 6, 7, and 8 show the surviving fractions, double strand breaks, and single strand breaks per million base pairs for all the experiments in this study. Surviving fractions are reported as colony forming units (CFU); the number of colonies that grew on an agar plate. Total numbers of colony forming units per sample were divided by the original number of viable spores per sample, 5×10^9 spores, to obtain a surviving fraction.

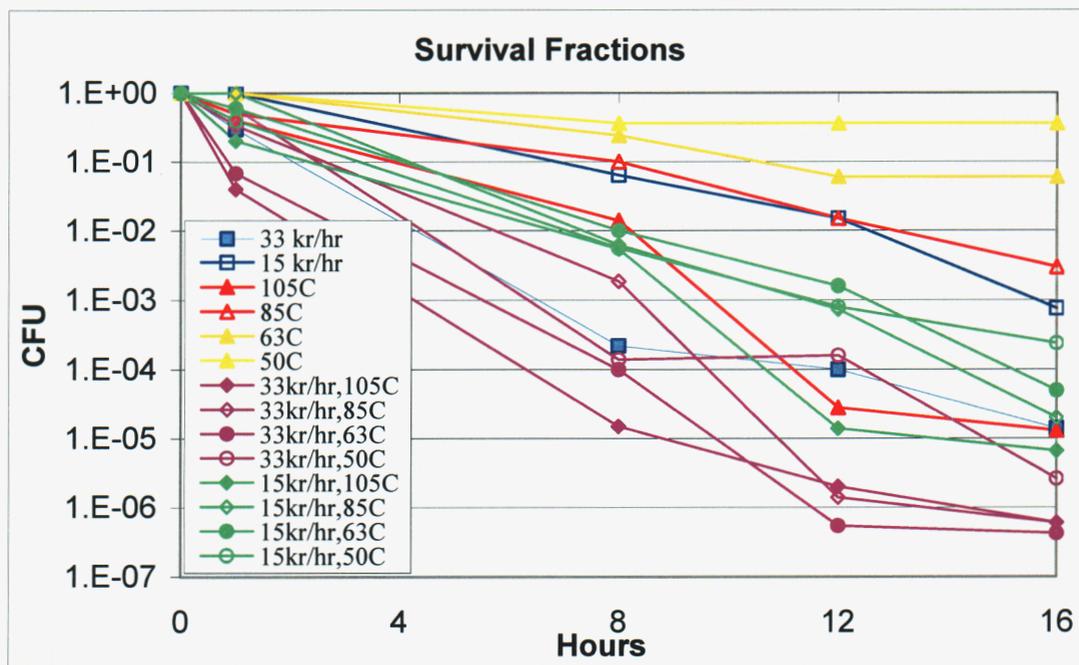


Figure 6. Survival fractions of all experiments conducted, given in colony forming units vs. hours treated.

Because data collection for this study was extremely time-intensive, the points shown on all graphs represent only one experimental data point. For this reason, as well as the great difficulty in determining the error associated with the biological procedures described in the Methods section, error bars are not shown on any of the graphs of data results. Therefore, data trends, rather than individual data points, should be given more weight in examining these experimental results. Clearly, repeating the experiments performed in this study would be a useful extension of this work.

From Figure 6 it is apparent that in combination with 33 kr/hr of radiation for 12 or 16 hours, it does not much matter whether one uses heat at 63° C, 85° C, or 105° C, as the surviving fraction after these times is essentially the same. Only when the temperature is dropped to 50° C does the surviving fraction increase, and this increase is not great. Interestingly, it does seem that there is a

significant difference at 8 hours in the values of these four survival fractions. However, at this 8 hour point, the greatest surviving fraction comes from the thermoradiation treatment with 85° C, not 50° C, as might be expected. The reason for this is not clear, as the most dramatic change of slope between 8 and 12 hours comes from the 33 kr/hr, 85° C combination—and yet the slope of the line depicting the effects of 85° C alone is remarkably consistent in this range. It may be that more measurements at this 8 hour point for the 33 kr/hr, 85° C would show this measurement to be an outlier.

Figure 7 shows the magnitude of double strand breaks per million base pairs caused by each of the various combinations of heat and radiation examined. From the three combinations of energies that were most effective in killing spores--33 kr/hr in combination with 105° C, 85° C, and 63° C--we cover both extremes of the double strand breakage chart; 33 kr/hr with 105° C is very high in double strand breaks, while 33 kr/hr with 85° C and 33 kr/hr with 63° C are both very low in double strand breaks. This seems to indicate that these double strand breaks may not be the direct cause of the spore death. Interestingly, 33 kr/hr with 50° C caused a fair amount more double strand breaks than did 33 kr/hr in combination with 85° C or 63° C.

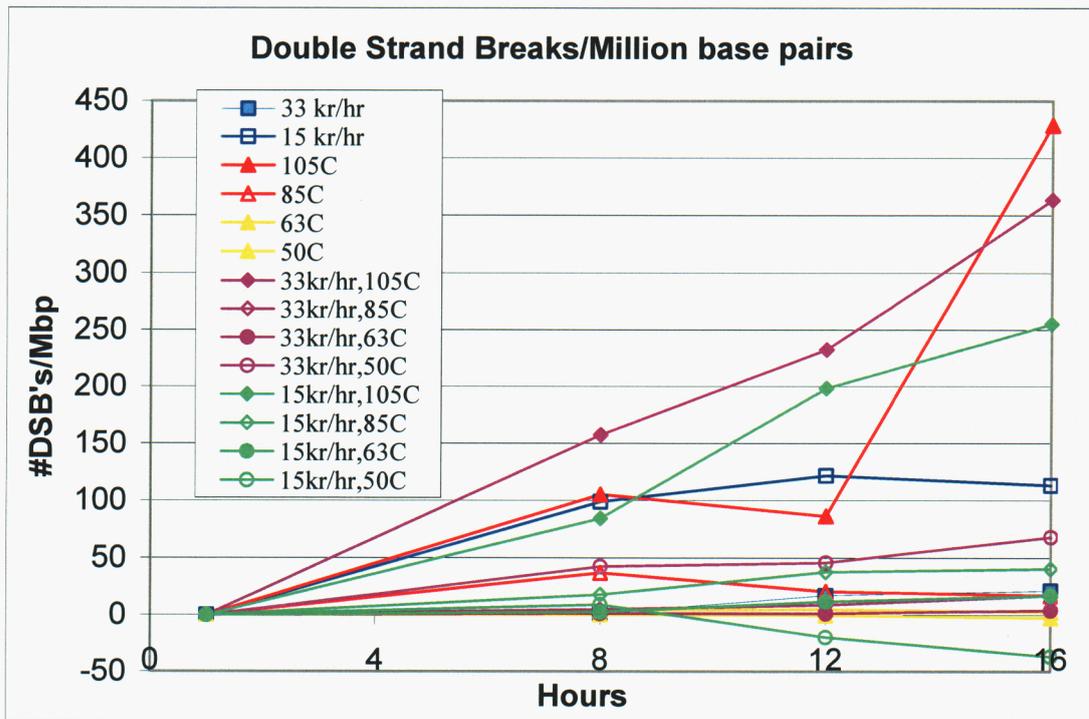


Figure 7. Double strand breaks per million base pairs of DNA for all experiments conducted.

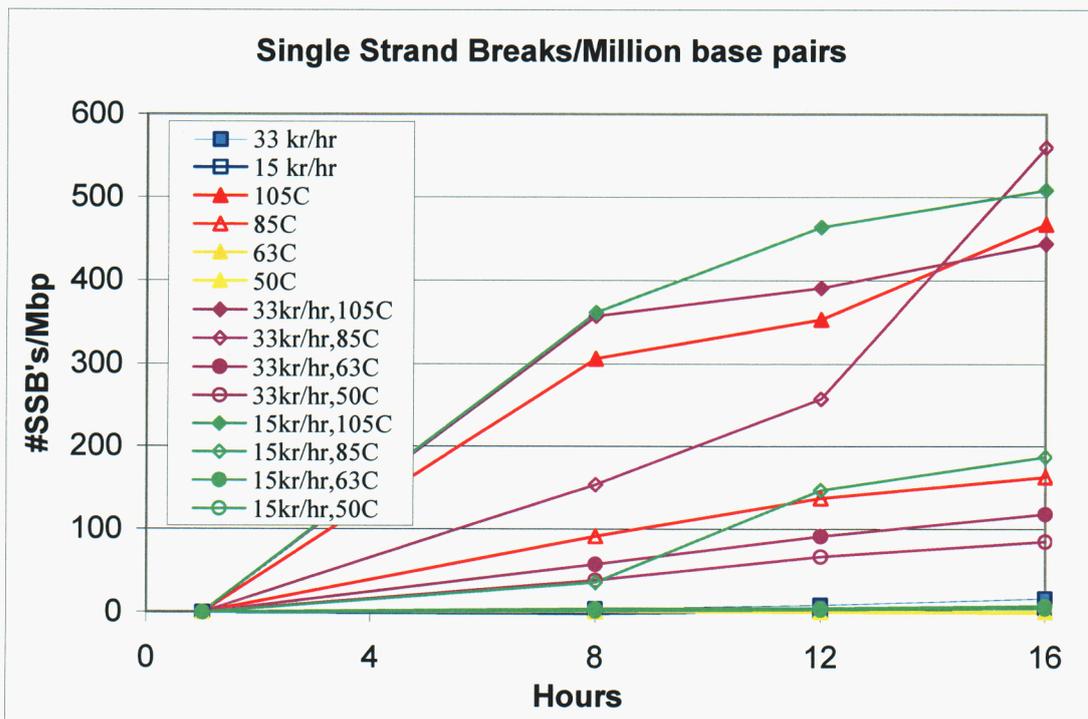


Figure 8. Single strand breaks per million base pairs of DNA for all experiments conducted.

Figure 8 shows the magnitude of single strand breaks per million base pairs caused by each of the various combinations of heat and radiation examined. Again examining our three combinations most effective in cell killing, 33 kr/hr with 105° C is high in single strand breaks, 33 kr/hr with 85° C is also high, and 33 kr/hr with 63° C is moderately low. This also seems to indicate a lack of a direct link between single strand breaks and spore death. The combination of 33 kr/hr with 50° C, however, caused slightly fewer single strand breaks than did 33 kr/hr with 63° C, as might be expected.

A comparison of the data for 105° C with each of the two radiation levels shows that in combination with 33 kr/hr more spores were killed than when in combination with 15 kr/hr, as seems logical. More double strand breaks are seen when 105° C is in combination with the higher radiation level than when in combination with the lower level, also as might be expected. However, 33 kr/hr with 105° C did not have more single strand breaks than 15 kr/hr with 105° C.

Similarly, comparing the data for 85° C with each of the two radiation levels reveals that the higher radiation at 85° C had a greater killing effect, and caused more single strand breaks, but fewer double strand breaks. This is interesting in that it is the reverse of the situation seen for 105° C in combination with each of the radiation levels, giving contrary indications of which type of strand breakage, if either, is an indicator of cell death.

If we perform the same comparisons for the two radiation levels in combination with 63° C, we find that 33 kr/hr with 63° C had a significantly greater killing effect than 15 kr/hr with 63° C, and caused significantly greater single strand breaks, but the number of double strand breaks from the two thermoradiation combinations were approximately equal, with 15 kr/hr, 63° C having a slightly higher number of double strand breaks. This situation parallels that which we saw when we compared the two radiation levels in combination with 85° C.

Looking at the final temperature, 50° C, in combination with each level of radiation, we see what might have been expected: the higher radiation level combined with 50° C had a greater killing effect, and caused greater numbers of strand breaks, both single and double.

A comparison of 15 kr/hr in combination with 105° C and 85° C finds that the higher heat combination killed marginally more spores, but it caused many more double and single strand breaks. In a similar manner, 15 kr/hr with 63° C and with 50° C each left incrementally greater surviving fractions. However, the number of double strand breaks decreases less dramatically, and the number of single strand breaks for the two combinations, 15 kr/hr with 63° C and with 50° C, is essentially equal. This value is significantly less than the number of single strand breaks from 15 kr/hr, 85° C. For this set of data, both double and single strand break numbers are ordered to fit with survival fraction data; the

inconsistency is in proportion; although the surviving fraction increases as the temperature decreases, the number of strand breaks do not decrease at a similar rate.

Heat alone follows a pattern somewhat similar to that of 15 kr/hr in combination with each temperature. Higher heat consistently caused greater cell death, along with more double and single strand breaks. However, the number of strand breaks from 105° C is vastly greater than the number of breaks caused by the lower temperatures. It is interesting to compare the numbers of strand breaks for each temperature alone with the values for that temperature in combination with 15 kr/hr. With the exception of the number of double strand breaks at 105° C alone, these values are relatively similar, with the thermoradiation causing slightly higher numbers of single strand breaks. The plot of double strand breaks for 105° C is rather choppy, and may reflect an outlier that would be discovered through additional measurements. This aside, the similarity of strand breaks between heat alone and the same temperatures combined with 15 kr/hr suggests that this rate of radiation is low compared to the temperatures involved, and the temperatures are essentially dictating the strand breakage. If we look, though, at the chart showing survival fraction, we see that despite these similar strand breakage values, the addition of radiation to each temperature resulted in a significant increase in cell death, with the exception of 105° C, in which the increase in cell death is minimal. The mechanism involved appears to significantly increase cell death when radiation is combined with the heat, while only slightly increasing strand breaks.

The results for radiation alone were less than intuitive. The higher dose rate caused greater cell death, but caused fewer double strand breaks, while the numbers of single strand breaks from the two dose rates were approximately equal. These results for the radiation treatments alone seem to imply that radiation-induced strand breaks do not lead directly to cell death.

Table 2 gives the statistical parameter of correlation, r , for each of the various combinations of energy used in this study. This parameter measures, for a series of two variables x and y , how strongly x is related to y ; in this case r is measuring a linear correlation. A conventional rule of thumb is to consider the correlation weak if $0 \leq |r| \leq 0.5$, strong if $0.8 \leq |r| \leq 1$, and moderate otherwise (Devore, 1987). Values in Table 2 are **bold** if they represent strong correlations, and *italicized* if they represent weak correlations.

Table 2. Correlations ($|r|$) of survival fraction (SF) with double and single strand breaks for all treatments. Strong correlations are in **bold**, weak correlations are in *italics*.

Treatment	$ r $ (SF with SSB)	$ r $ (SF with DSB)
33 kr/hr only	0.8565	0.7686
15 kr/hr only	0.8182	0.8227
105° C only	0.9003	0.7128
85° C only	0.9616	<i>0.2830</i>
63° C only	0.5433	<i>0.4480</i>
50° C only	<i>0.4690</i>	<i>0.4431</i>
33 kr/hr, 105° C	0.9941	0.9403
33 kr/hr, 85° C	0.8803	0.9087
33 kr/hr, 63° C	0.9803	0.7505
33 kr/hr, 50° C	0.9439	0.9977
15 kr/hr, 105° C	0.9242	0.9898
15 kr/hr, 85° C	0.9513	0.9575
15 kr/hr, 63° C	0.9916	0.9465
15 kr/hr, 50° C	0.9779	0.7222

The correlation values shown in Table 2 should allow us to determine whether the number of double or single strand breaks under the given conditions can be used to predict the survival fraction for *B. subtilis* under those same conditions. Clearly we can do this with single strand breaks for all of the given conditions except the lowest two heat treatments, 63° C and 50° C. This seems a good indication that either single strand breaks are significant in the killing of the spore or that whatever mechanism is responsible for killing the spore also causes single strand breaks in similar proportions.

The correlation of the survival fractions with double strand breaks are a somewhat different story. With the exception of 33 kr/hr in combination with 63° C, and 15 kr/hr in combination with 50° C, all of the thermoradiation treatments showed excellent correlation between the survival fraction and the number of double strand breaks. Individually, however, only the lower radiation dose rate, 15 kr/hr, showed a strong correlation, while the three lowest heat values, 85° C and 63° C, and 50° C, showed weak correlations. Although 33 kr/hr showed only a moderate correlation between dsb and survival, Table 2 does seem to indicate that the correlation for ionizing radiations alone are stronger than those for heat alone, while the correlations for thermoradiation treatments are quite strong in general. Heat alone, in fact, seems to have rather little correlation between dsb and survival.

A. Data for 33 kr/hr, 63° C

Figures 9, 10, and 11 show the data obtained for 33 kr/hr, 63° C, and the combination of these two energies. In addition, a line showing the hypothetical results of the addition of these two individual energies is given. This line is simply the mathematical addition of the trend lines for radiation at 33 kr/hr and heat at 63° C. If actual experimentation using the two energies in combination obtains results greater than those predicted by this hypothetical line, the difference is considered the amount of synergism. In Figure 9, we see a synergistic spore killing effect. However, the combination of radiation and heat energies seems to have decreased the number of double strand breaks (Figure 10), while extensive synergism of single strand breakage is seen from Figure 11.

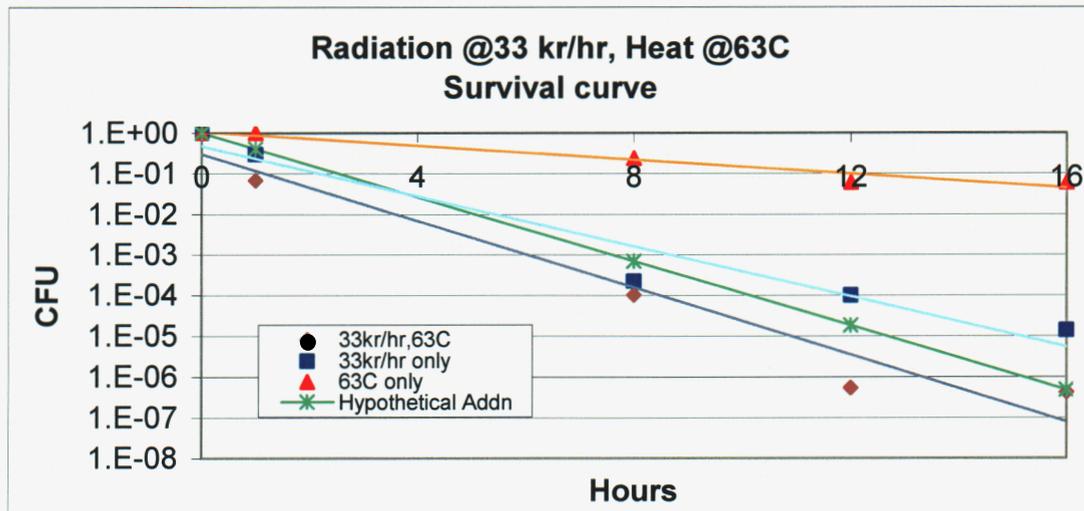


Figure 9. Survival fractions for a radiation dose rate of 33 kr/hr and heat at 63° C, given in colony forming units.

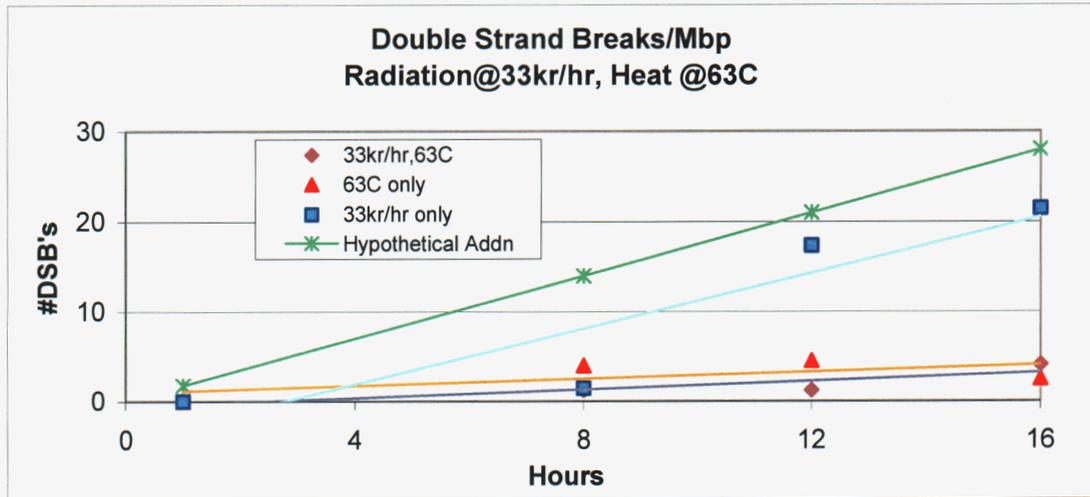


Figure 10. Double strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 63° C.

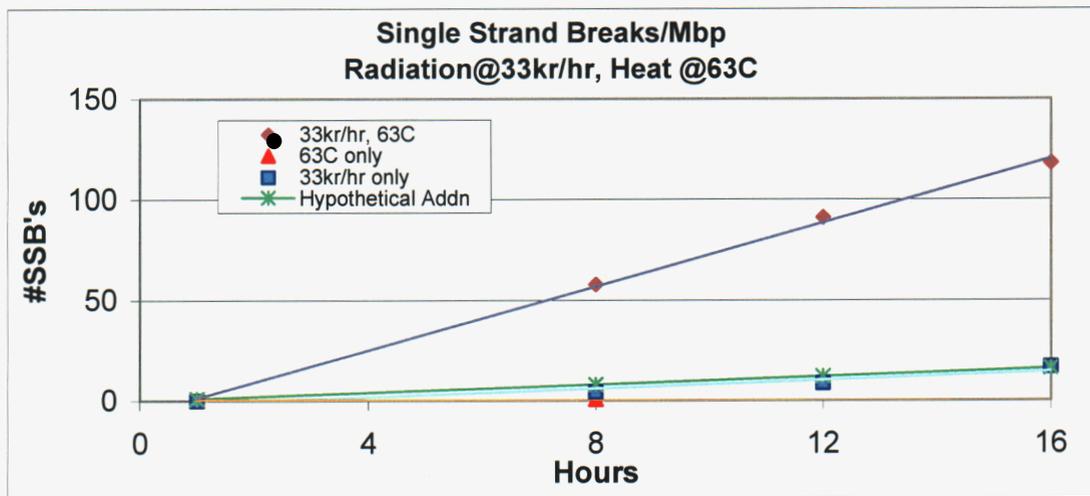


Figure 11. Single strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 63° C.

It is very possible that the heat disrupts the hydrogen bonds holding the double helix together. This bond is illustrated in Figure 12.

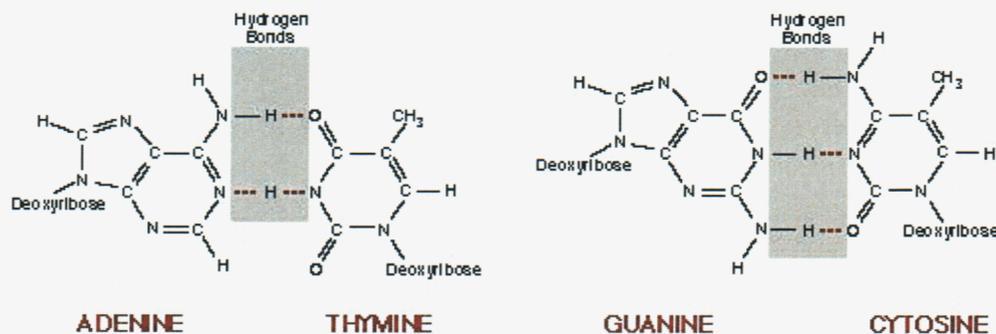


Figure 12. Hydrogen bonding between strands of DNA.

The heat energy required to break such a hydrogen bond is only 4.2-8.4 kJ/mol, making it very likely that this breakage occurs in significant numbers. Although one possibility is that this loss of hydrogen bonding provides a larger target for radiation and thus explains the thermoradiation synergistic effect, this explanation does not seem likely. Goodhead (1994) has calculated that the free radicals and ions produced by ionizing radiation in cells have only nanometer diffusion distances. This is a fairly small distance in comparison to the spore, which is several hundred times larger than this in diameter. Therefore the free radicals and ions do not travel far enough to make a small change in target size significant. If the radiation energy is not initially deposited relatively near the DNA strand, the subsequent radicals and ions will not reach the DNA. A more significant effect from the loss of hydrogen bonding between the two DNA strands is that the deposited radiation energy affects only one strand rather than both. Because hydrogen bonds are responsible for keeping the double helix double, breaking the hydrogen bonds leaves the two DNA strands essentially single. This melting of the double-stranded DNA is known as DNA denaturation. For *B. subtilis*, denaturation occurs in the vicinity of 87° C. However, the regions that are rich in adenine-thymine pairs will melt at lower temperatures than regions with greater proportions of guanine-cytosine pairs, because A-T pairs have only 2 hydrogen bonds, while G-C pairs have 3 hydrogen bonds. Therefore, some portions of the DNA strand will denature at lower temperatures than the strand as a whole. These single strands of DNA then suffer individual breaks from both the heat energy and the radiation energy. Radiation energy deposited near one strand is no longer as likely to break both strands simply because the second strand is not nearby, having been released from the hydrogen bonds that would have otherwise held it close to the first strand. Normally denaturation of DNA is reversible; once the heat is removed, hydrogen bonds reform. However, if strand breaks occur while the DNA is denatured into the single-strand state, this would make proper reformation difficult. Because the broken fragments are no longer anchored by hydrogen bonds to their matching strands, they are left "floating" and do not return to their original positions once the treatment is finished and hydrogen bonds could be reconstructed. Hydrogen bonds that are reconstructed may occur in improper positions, thus creating even more difficulty for repair enzymes. This reasoning would call for a decrease in double strand breaks with thermoradiation, and a

corresponding single strand break increase. The result of this situation is that strand breaks created during thermoradiation are less repairable, and so more fatal, than those caused by either heat or radiation alone.

An examination of Figures 10 and 11 supports this mechanistic theory. Radiation alone in Figure 10 created over 20 double strand breaks per million base pairs. When used in combination with heat, the double strand breaks drop to less than 5 breaks per million base pairs. At the same time the single strand breaks have increased from approximately 20 single strand breaks per million base pairs due to radiation alone, up to 120 ssb per million base pairs when this same radiation is combined with the heat.

B. Data for 33 kr/hr, 50° C

Figures 13, 14, and 15 show the data obtained for 33 kr/hr, 50° C, and the combination of these two energies, as well as the aforementioned line indicating hypothetical addition of effects. From Figure 13 we can see that a minute amount of synergism in spore killing was observed. Looking at Figure 14 we see that there is a fairly large amount of synergism in the double strand breaks. In Figure 15, showing single strand breakage, there is a very large synergistic effect from the radiation and heat combination. From this data it is difficult to determine why there is such a large synergism in the double strand breakage. However, it is very likely that 50° C is simply not enough energy to separate the strands of DNA sufficiently to prevent recombination when the heat is removed. As discussed earlier, an examination of Figure 6 shows that 33 kr/hr, 50° C actually resulted in a greater number of double strand breaks than the same radiation level in combination with 63° C or 85° C. This is probably because 63° C and 85° C have caused strand separation, while 50° C has not.

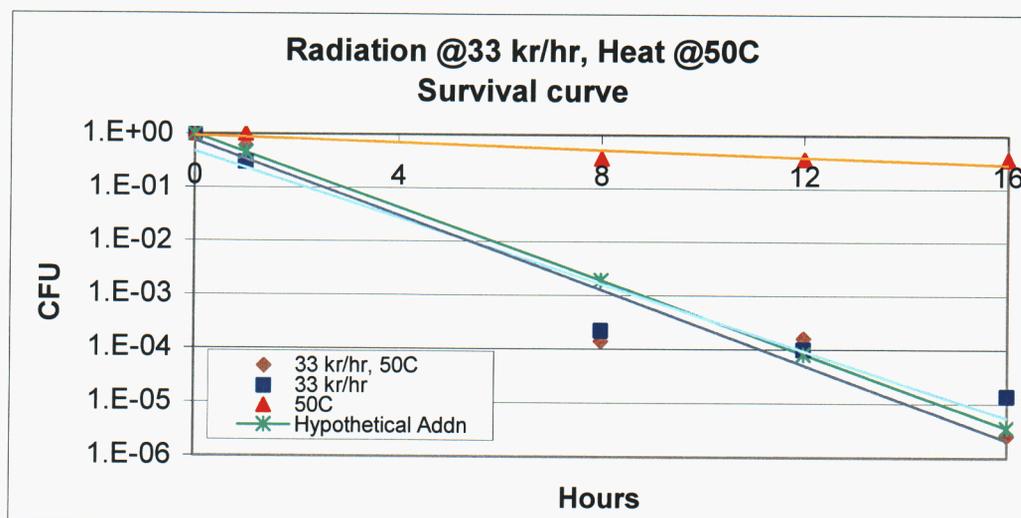


Figure 13. Survival fractions for a radiation dose rate of 33 kr/hr and heat at 50° C, given in colony forming units.

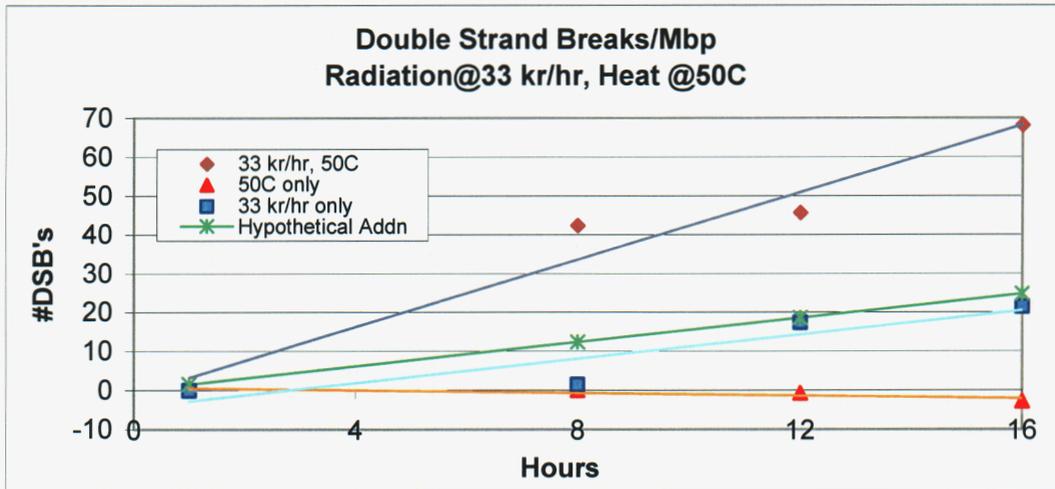


Figure 14. Double strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 50° C.

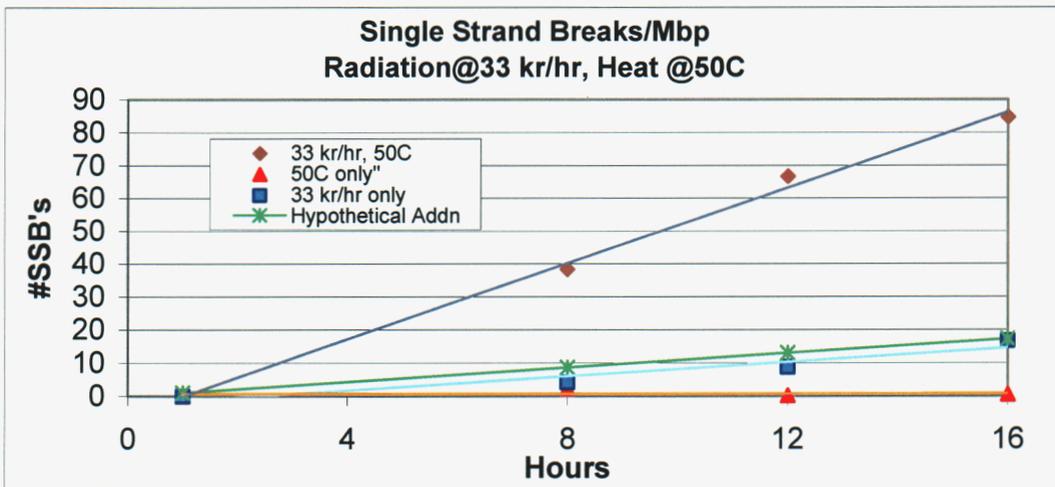


Figure 15. Single strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 50° C.

C. Data for 33 kr/hr, 85° C

Figures 16, 17, and 18 show the data obtained for 33 kr/hr, 85° C, and the combination of these two energies, as well as the line indicating hypothetical addition of effects. From Figure 16 we can see that no synergism in spore killing was observed. Looking at Figure 17 we see that again combining the radiation and heat energies seems to have decreased the number of double strand breaks incurred. In Figure 18, showing single strand breakage, there is a significant synergistic effect from the radiation and heat combination. It appears from the loss of double strand breaks in the thermoradiation treatment and the synergistic gain of single strand breaks from thermoradiation that this data set

might show synergism in spore killing—but it does not. The explanation for this may be that although the single strand breaks did display synergism from thermoradiation, it simply was not great enough to translate into spore killing synergism. Sivinski (1972) observed that too much of one energy, heat or ionizing radiation, in comparison to the other, will negate the synergism in spore killing. The higher energy seems to simply overwhelm the other and so no synergism is observed. A comparison of Figures 9-11 and Figures 16-18 shows that the major difference in strand breaks, both double and single, is due to the much larger effect on strand breaks that 85° C had versus 63° C. The survival curves in Figures 9 and 16 also show that 85° C was much more effective in killing spores than was 63° C. And yet in combination with the same dose of ionizing radiation, the spore survival rates are essentially identical! Many more DNA strands are broken by the 33 kr/hr, 85° C thermoradiation than are broken by the 33 kr/hr, 63° C thermoradiation, while again, survival rates are the same. It seems then that the greater number of strand breaks (both single and double) from the 33 kr/hr, 85° C thermoradiation must be attributed to the greater effect from the heat. Since this greater number of strand breaks did not translate into greater spore killing, these strand breaks attributable to heat, within the thermoradiation treatment, must be less fatal in some way, or perhaps just more easily repaired. This study did not distinguish between one double (or single) strand break and another in terms of position on the DNA strand, or functionality of the area involved. It may be that these factors are critical in determining the effect of the particular break on the spore. Goodhead's argument that not all double strand breaks are identical in nature or effect on the cell, and that such division of breaks into only double or single strand breaks is too simplistic may be applicable here (1994). If this is the case, then further evaluation of the exact type of double strand breaks and of single strand breaks may be necessary to better evaluate the mechanism responsible for the synergism observed from thermoradiation.

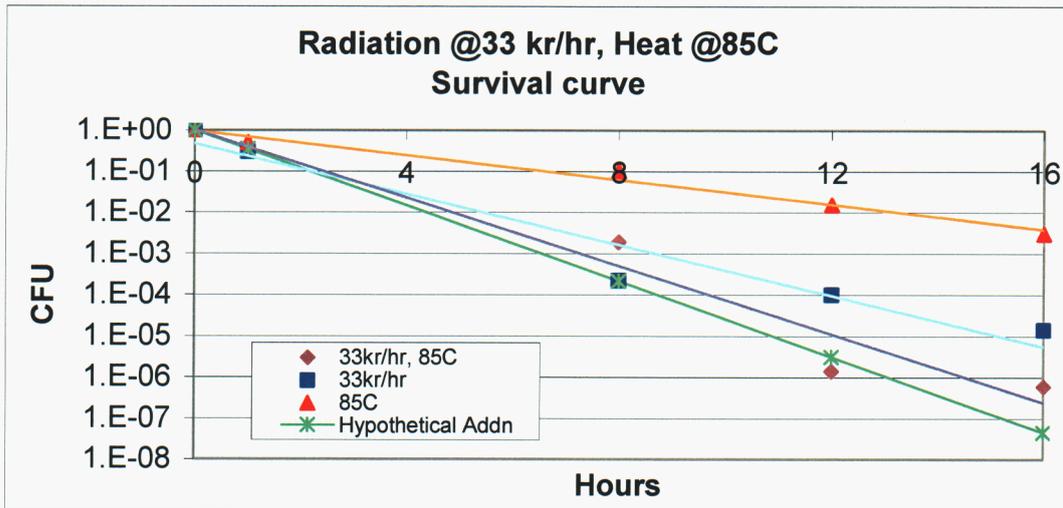


Figure 16. Survival fractions for a radiation dose rate of 33 kr/hr and heat at 85° C, given in colony forming units.

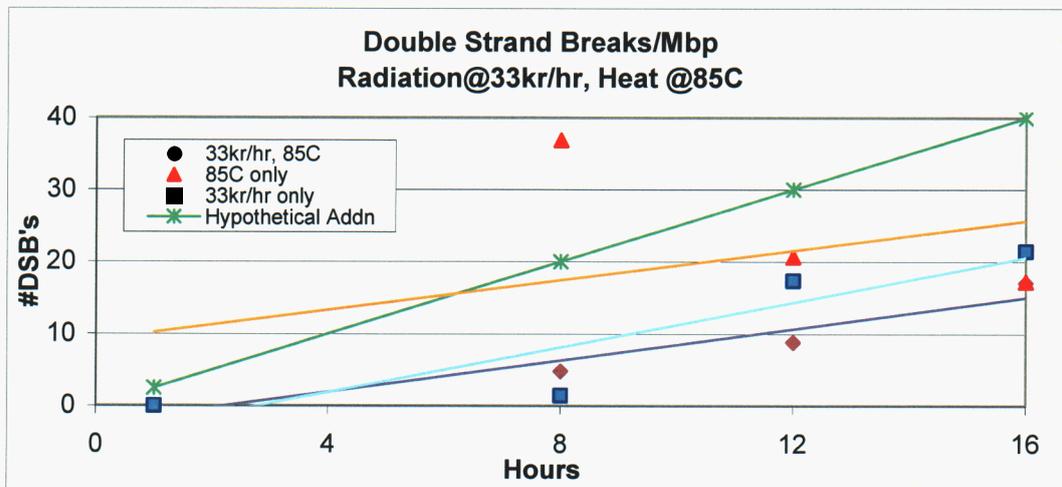


Figure 17. Double strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 85° C.

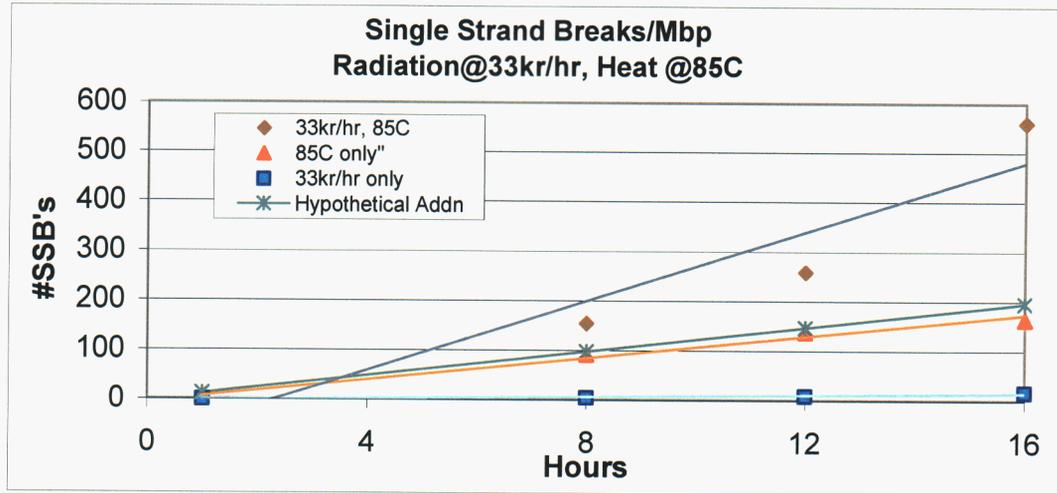


Figure 18. Single strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 85° C.

D. Data for 33 kr/hr, 105° C

Figures 19, 20, and 21 show the results of an overwhelming amount of heat energy in combination with the same radiation dose rate examined above. This data is for a radiation dose rate of 33 kr/hr, heat at 105° C, and the two energies combined. From Figure 19 we can see that synergism in spore killing was not observed from the 33 kr/hr, 105° C thermoradiation. Synergism was not observed from this energy combination in the double or single strand breakage either. The values observed for double strand breaks inflicted by heat energy, shown in Figure 20, are rather randomly scattered at the points for 8, 12, and 16 hours, seemingly indicating that after 8 hours of heating, there is no further proportional change in double strand breakage. This observation is consistent at every heat tested in this study (see also Figures 10 and 17). Also consistent throughout this study at every heat energy was a very proportional increase in the number of single strand breaks with increased time of treatment (see Figures 11, 18, and 21). This observation seems to indicate very clearly that heat causes single strand breaks in *B. subtilis* spores. Whether or not these single strand breaks are the primary cause of spore death is another matter. Values of correlation, r , from Table 2 suggest that the single strand breaks at 105° C and 85° C may be strongly related to the cause of spore death, while perhaps this is not the case for single strand breaks at 63° C.

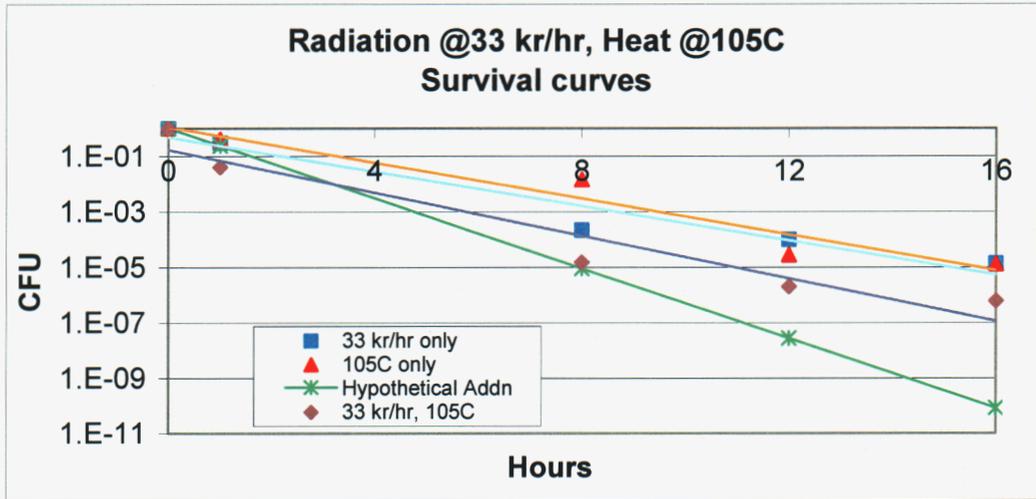


Figure 19. Survival fractions for a radiation dose rate of 33 kr/hr and heat at 105° C, given in colony forming units.

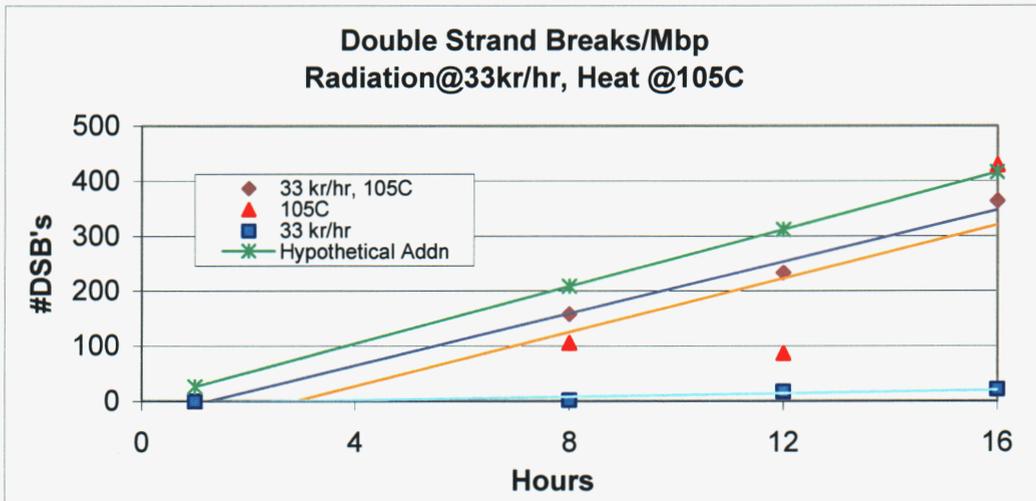


Figure 20. Double strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 105° C.

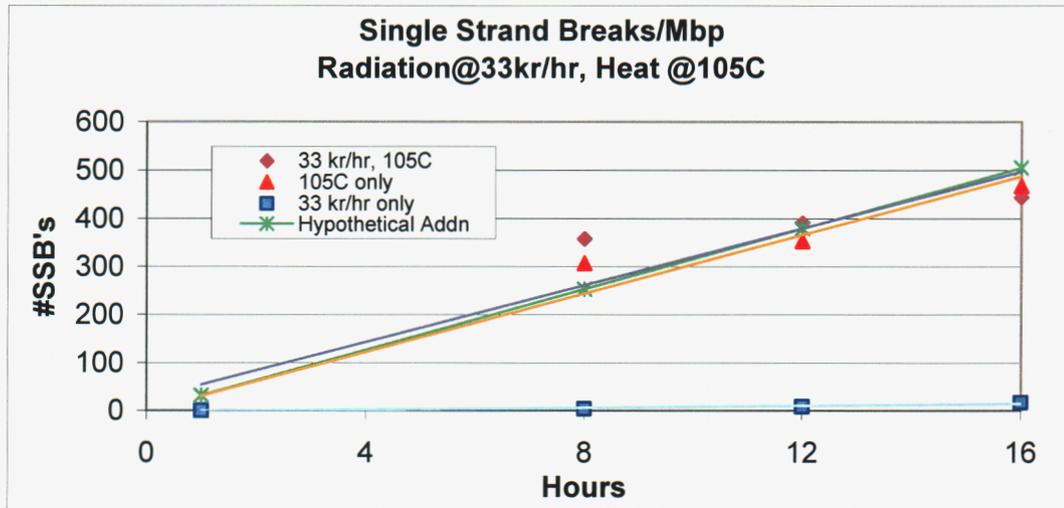


Figure 21. Single strand breaks per million base pairs of DNA for a radiation dose rate of 33 kr/hr and heat at 105° C.

E. Data for 15 kr/hr, 105° C

Let us now examine heat combinations with 15 kr/hr of ionizing radiation. Figures 22, 23, and 24 show the data for 15 kr/hr, 105° C, and the combination of these two energies.

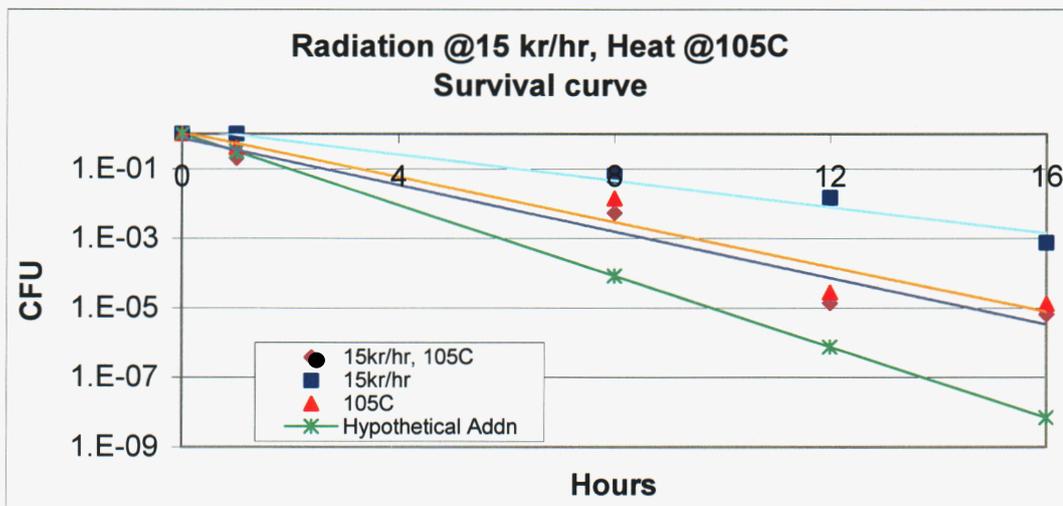


Figure 22. Survival fractions for a radiation dose rate of 15 kr/hr and heat at 105° C, given in colony forming units.

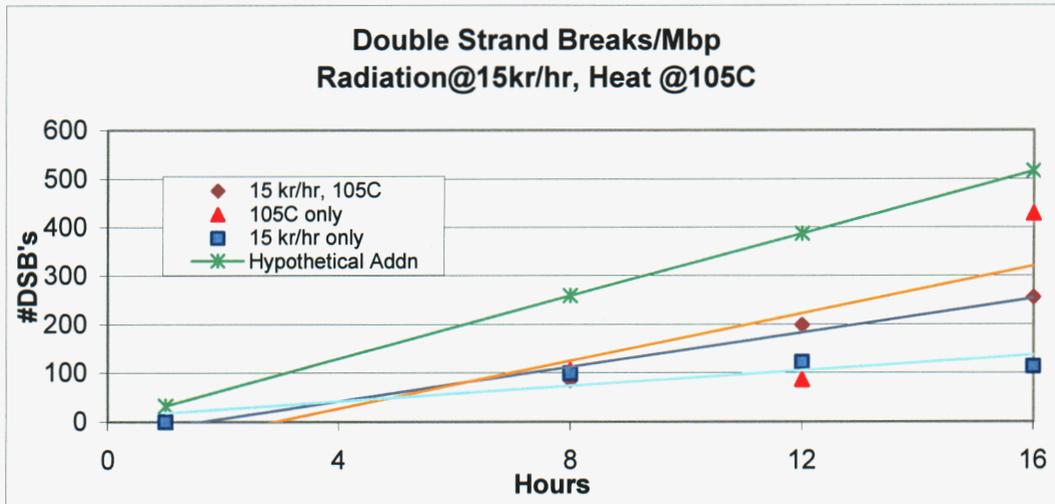


Figure 23. Double strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 105° C.

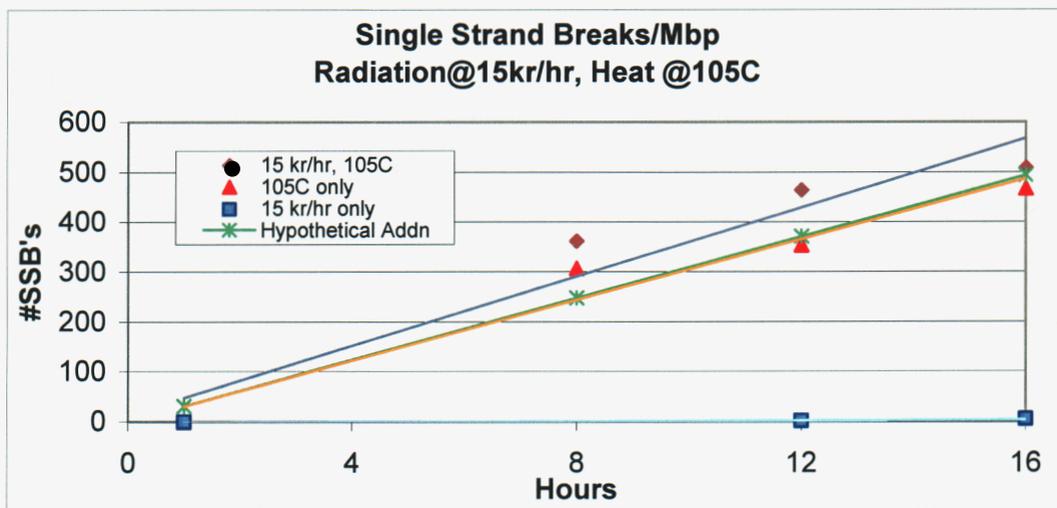


Figure 24. Single strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 105° C.

No synergism in spore killing is seen in Figure 22; Figure 23 shows a reverse synergistic effect in double strand breakage, and Figure 24 reveals a small amount of synergism in single strand breakage. Comparing this energy combination (15 kr/hr, 105° C) with the same temperature at the higher radiation dose rate (33 kr/hr, 105° C—see Figures 19-21) the number of double strand breaks from 15 kr/hr alone is greater than that from 33 kr/hr alone. However, in combination with 105° C, thermoradiation with 15 kr/hr had fewer double strand breaks than thermoradiation with 33kr/hr. The radiation at 15 kr/hr is 5.4 times more effective at creating double strand breaks (evaluated from the 16-hour data points) than radiation at 33 kr/hr, but in combination with the same heat energy, the radiation at 15 kr/hr is only 0.7 times as effective at creating double

strand breaks. Performing a similar analysis with single strand breaks, we find that 15 kr/hr alone creates only 0.33 times as many single strand breaks as 33 kr/hr alone, but thermoradiation with 15 kr/hr, 105° C, creates 1.1 times as many single strand breaks as thermoradiation from 33 kr/hr, 105° C. This is in agreement with the hypothesis that combining heat energy with the radiation makes double strand breaks more rare, while single strand breaks become more plentiful, since the strands are not as likely to be held together by hydrogen bonding. However, the overall effect in this data set is that the heat is again overwhelming the radiation, and controlling the outcome, both in strand breaks and spore killing.

F. Data for 15 kr/hr, 85° C

Figures 25, 26, and 27 show the data obtained for 15 kr/hr in combination with 85° C.

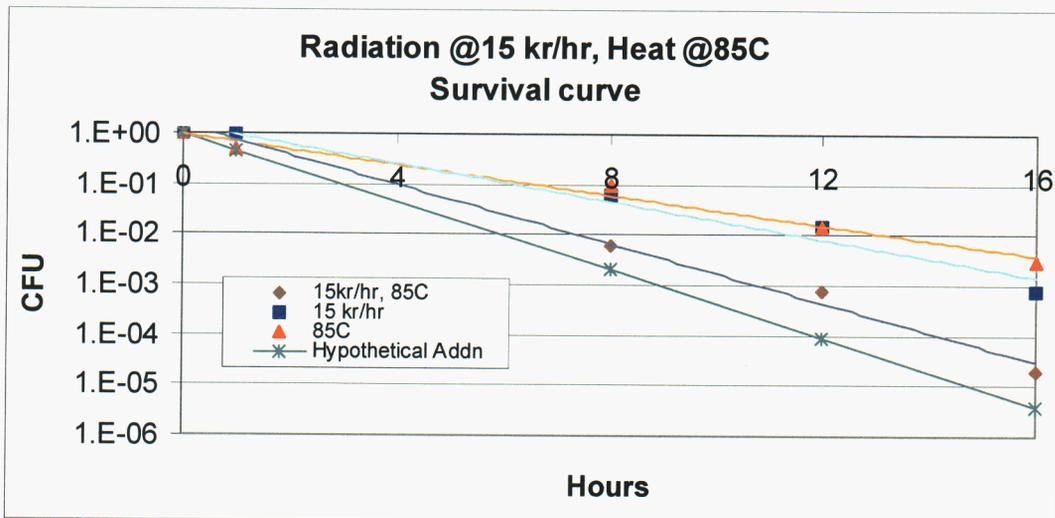


Figure 25. Survival fractions for a radiation dose rate of 15 kr/hr and heat at 85° C, given in colony forming units.

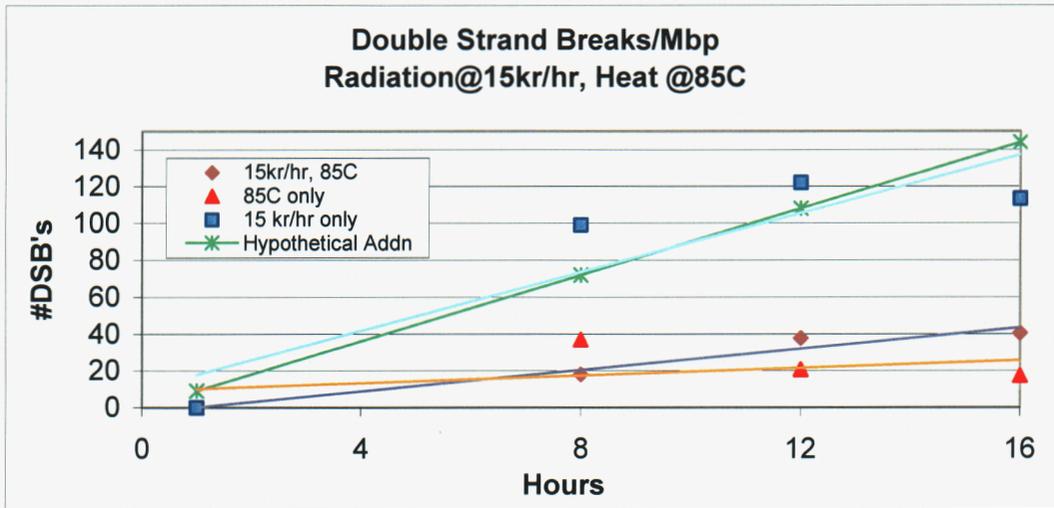


Figure 26. Double strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 85° C.

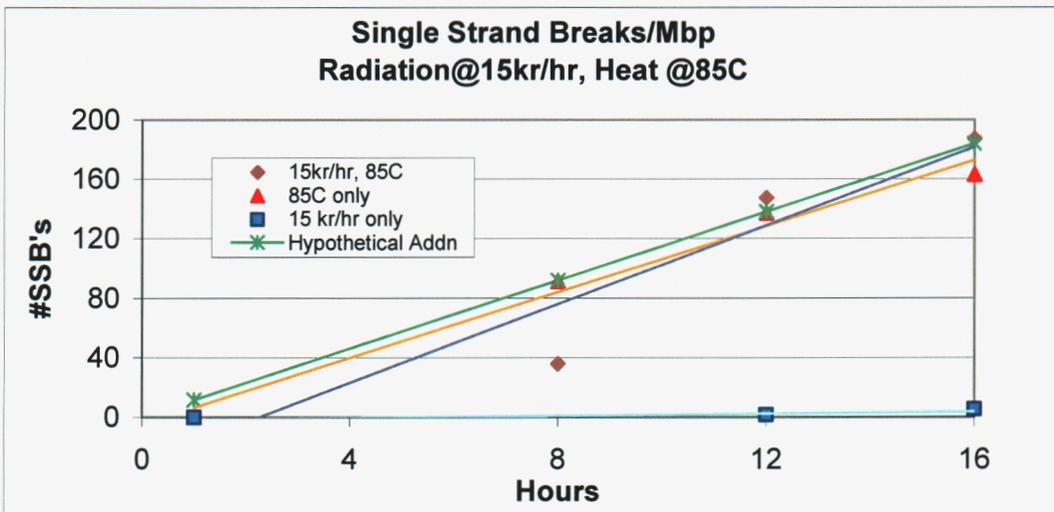


Figure 27. Single strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 85° C.

Here we see no spore killing synergism (Figure 25), while there is reverse synergism in both double and single strand breaks (Figures 26 and 27). It appears in this case that although the 85° C temperature was able to separate the DNA strands, the two energies were not enough to cause enough strand breaks to results in a synergistic killing effect. Similarly, Figures 28, 29, and 30 show that 15 kr/hr in combination with 63° C was also unable to cause sufficient damage to result in a synergistic killing effect.

G. Data for 15 kr/hr, 63° C

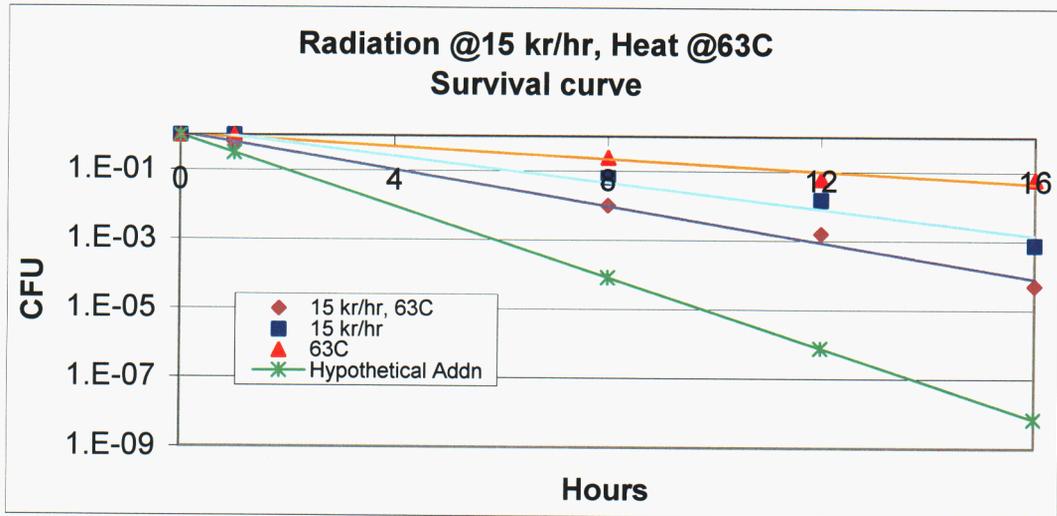


Figure 28. Survival fractions for a radiation dose rate of 15 kr/hr and heat at 63° C, given in colony forming units.

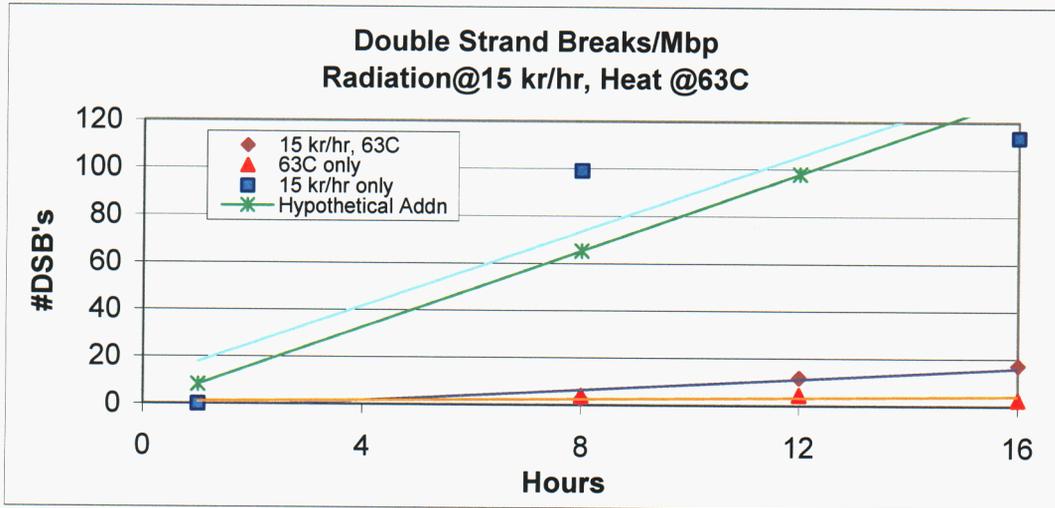


Figure 29. Double strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 63° C.

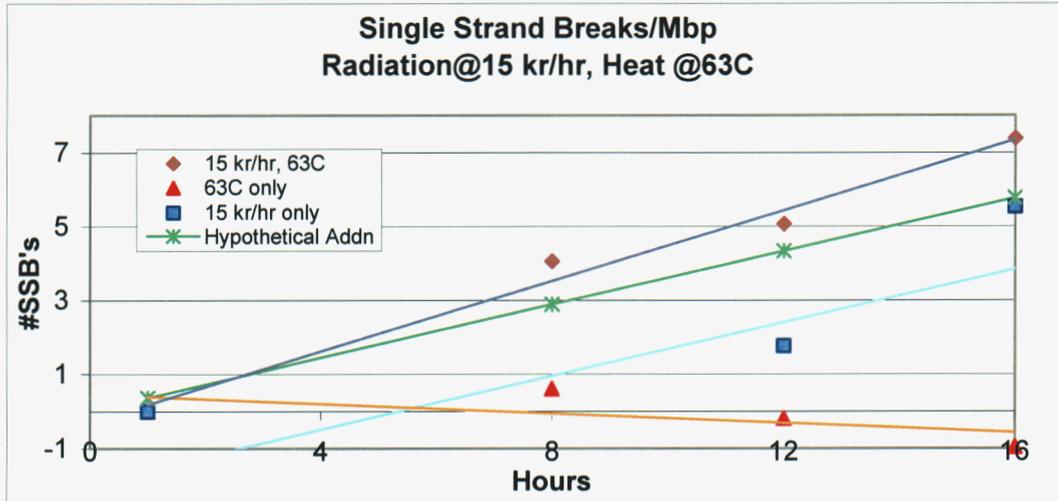


Figure 30. Single strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 63° C.

As Figure 29 shows, the reverse synergism of double strand breaks in this case was quite large, and a small amount of positive synergism appears in the single strand breaks (Figure 30). Lowering the heat once again, however, we finally have a combination that results in a synergistic killing effect at this radiation level.

H. Data for 15 kr/hr, 50° C

Figures 31, 32, and 33 show the results for 15 kr/hr in combination with 50° C. The synergism shown in Figure 31 (survival curve) is fairly small, but the reverse synergism of double strand breaks (Figure 32) is quite large. Figure 33, showing single strand breaks, reveals that in this case we do not have positive synergism of single strand breaks. This may be the reason for the small amount of synergism in spore killing that we see in this thermoradiation combination.

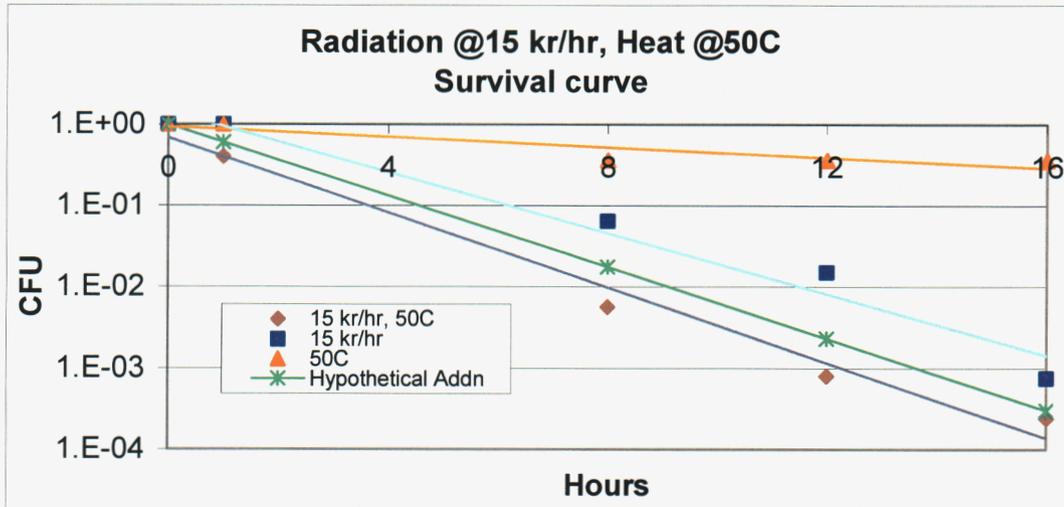


Figure 31. Survival fractions for a radiation dose rate of 15 kr/hr and heat at 50° C, given in colony forming units.

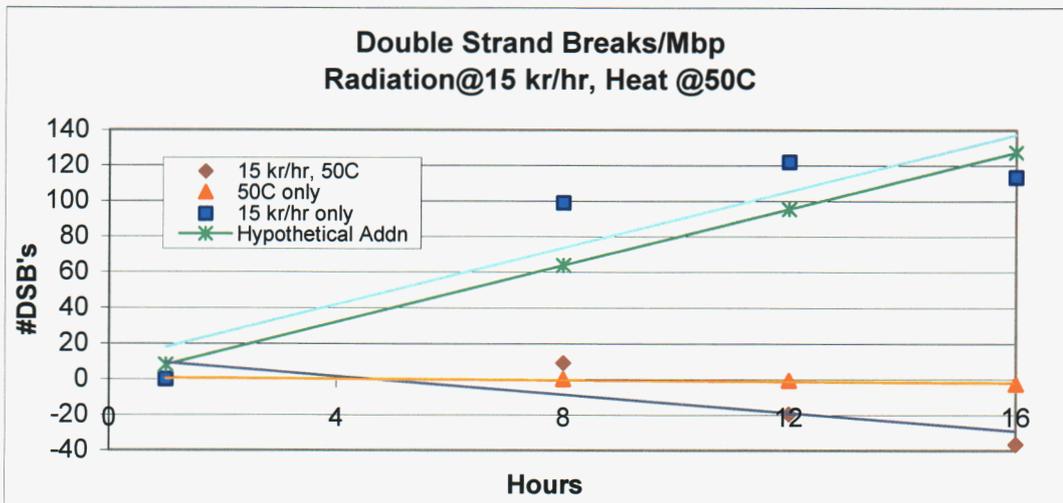


Figure 32. Double strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 50° C.

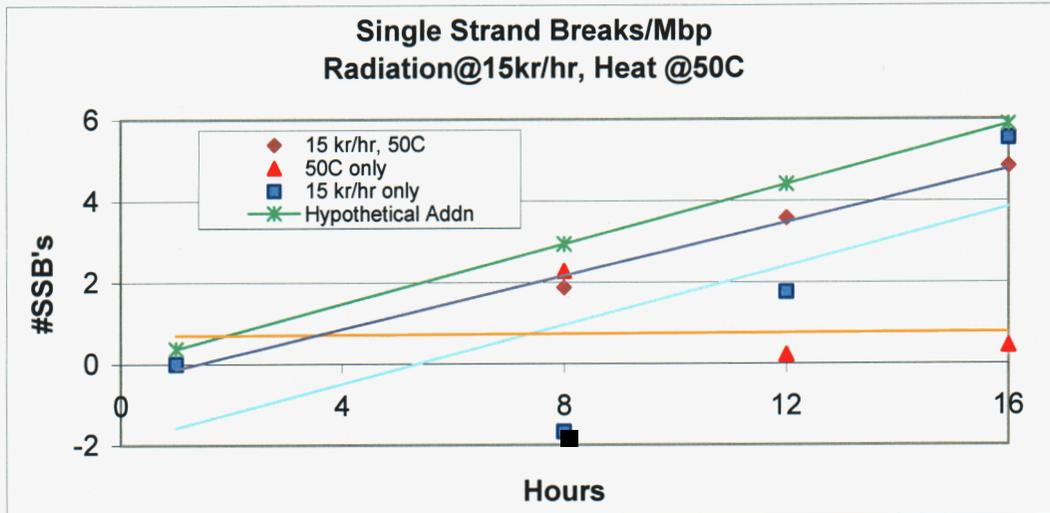


Figure 33. Single strand breaks per million base pairs of DNA for a radiation dose rate of 15 kr/hr and heat at 50° C.

I. Data for naked DNA treatment

In order to assess the effect of ions and free radicals housed by the rest of the spore on the DNA during irradiation treatments, a sample of naked DNA was irradiated at 33 kr/hr, then analyzed for strand breakage. The results of this treatment are shown in Figure 34. This data shows a greater rate of strand breakage for both single and double strand breaks in the naked DNA than was present for the DNA encased within the spore. Therefore it seems that the spore encasement has an overall protective effect against radiation for the DNA, rather than serving solely as a source of destructive free radicals and ions. It is likely that the free radicals and ions produced within the spore during irradiation do serve to damage the DNA, but clearly the radiation can damage the DNA without these free radicals and ions as well. However, it must be noted that although this naked DNA was thoroughly dried, some amount of H₂O remains indelibly complexed with the DNA itself. The effect of the radiation treatments on these fixed H₂O molecules is unknown. They may be a source of damaging free radicals and ions present in the irradiation of naked DNA.

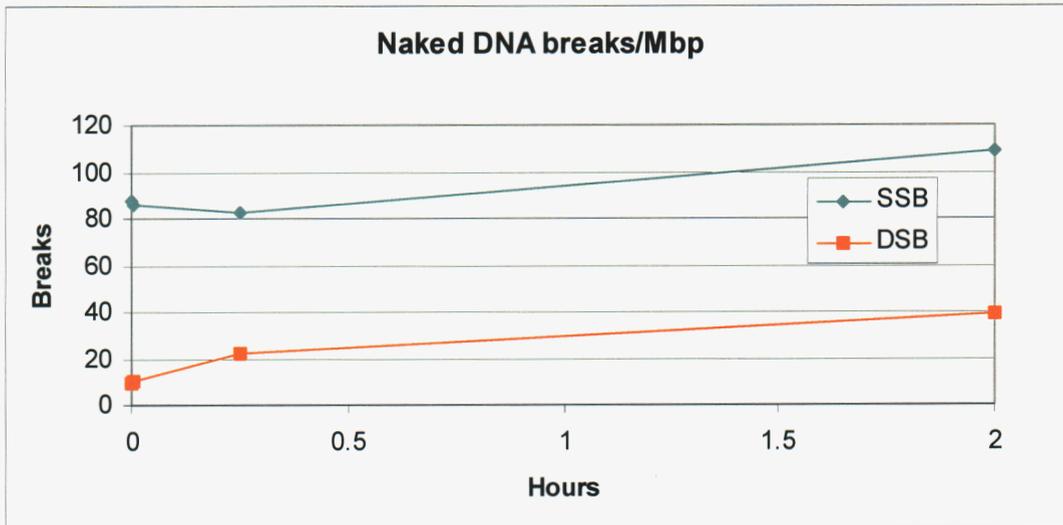


Figure 34. Strand breaks in naked DNA irradiated at 33 kr/hr

J. Data for enzymatic activity after treatment

Spores examined for enzymatic activity indicated that the enzymes responsible for DNA repair were active following all types of heat and radiation treatments. Nearly all the spores allowed 2 hours in 2xSG broth at 37° C showed additional growth rates from three to ten times those of spores similarly irradiated and heated, but not given the recovery time in incubation. The DNA of these spores allowed partial recovery was examined. Both single and double strand breaks had decreased in spores treated by irradiation only, at 33 kr/hr, and in spores treated by irradiation at 33 kr/hr in combination with heat at 63° C. Only the spores treated with heat only, at 63° C, showed no change in the number of DNA strand breaks. This is most likely because of the extremely low number of DNA strand breaks present from the 63° C treatment even in those spores not allowed 2 hours of recovery time.

V. Conclusions

The results of this work suggest that the synergistic killing effect on *B. subtilis* spores evident when particular levels of heat and radiation are combined is strongly correlated with single strand breaks, and may be partially correlated with double strand breaks in the DNA. However, a likely mechanism for the synergistic killing by thermoradiation—single strand breaks occurring during denaturation—has a more severe effect on the DNA than single strand breaks that occur while the double helix structure is maintained. Hydrogen bonds that reform after cessation of heating are likely to be incorrectly placed, thus leaving the various SSB's irreparable. Although this type of break may not reveal itself in gel electrophoresis as a double strand break, its effect on the spore's viability is probably similar. Table 3 summarizes the synergisms and correlations found in this study.

Table 3. Summary of synergisms in spore killing and in strand breaks, and of the correlations between cell killing and strand breaks at each thermoradiation combination evaluated.

Conditions	Synergism observed in each area?			Strong correlation with rate of spore killing (surviving fraction)?	
	Spore death	SSB	DSB	SSB	DSB
33 kr/hr, 50° C	Yes	Yes	Yes	Yes	Yes
33 kr/hr, 63° C	Yes	Yes	Reverse	Yes	No
33 kr/hr, 85° C	No	Yes	Reverse	Yes	Yes
33 kr/hr, 105° C	No	No	No	Yes	Yes
15 kr/hr, 50° C	Yes	No	Reverse	Yes	No
15 kr/hr, 63° C	No	Yes	Reverse	Yes	Yes
15 kr/hr, 85° C	No	No	Reverse	Yes	Yes
15 kr/hr, 105° C	No	Yes	No	Yes	Yes

As Table 3 shows, double strand breaks correlated strongly with spore killing at all thermoradiation treatments except the two exhibiting the most synergism of spore death (33 kr/hr with 63° C and 15 kr/hr with 50° C). This may be due to the denaturation effect discussed above. A third thermoradiation treatment, 33 kr/hr with 50° C also exhibited synergism in spore death and in this case the number of double strand breaks were strongly correlated with spore killing. However, the amount of synergism of spore death for this thermoradiation combination was extremely small. Spore killing from thermoradiation does appear to have a correlation to the number of DNA single strand breaks, although these single strand breaks are not definitively indicated as the cause of cell death.

Enzymatic repair activity was not broadly inhibited by the treatments applied, indicating that enzymes are not the primary target of thermoradiation. Naked DNA was more affected by radiation than DNA within the spores, indicating that free radical and ion action is also probably not the sole agent of thermoradiation spore death.

The strongest correlation found in this experimental work was clearly that of spore death with single strand breaks of the DNA. This is interesting, as single strand breaks are widely regarded as not a significant threat to cell viability. However, this work showed that in some cases, very large numbers of single strand breaks occurred, while the numbers of double strand breaks were relatively low. It is possible that the overwhelming number of single strand breaks, while not individually serious, are too much for the repair mechanisms of the cell to handle, even if these repair mechanisms are themselves functioning. The single strand breaks may also have occurred during a denatured state, allowing improper reformation of hydrogen bonding—and therefore DNA structure—following heating. Although this study showed that the repair enzymes were functioning following thermoradiation treatments, the examination of this aspect was not thorough and should be much more extensively examined to allow conclusive determination of the state of enzymatic repair activity. If enzymes are increasingly, even if only slightly, damaged from thermoradiation treatments, this coupled with the synergistically increasing number of single strand breaks could account for the synergistic spore killing observed.

VI. Future Work

There are several aspects of this work that should be further studied. First, all of the experiments performed in this study should be repeated, several times if possible. This is an extremely time-intensive requirement, which is why such a repetition of data points was not possible for this initial study. However, the additional data points would allow the development of experimental error bars, allowing a better evaluation of the accuracy of the data. In addition, further data points should be evaluated—points both in-between those examined in this study, as well as points extending to higher and lower values than those used in this study.

The areas of enzyme viability and the vulnerability of naked DNA versus DNA encased within a spore were only touched on in this study. Each of these areas could be the subject of extensive future work. Because enzymes are critical to the repair of DNA strand breaks, an experiment allowing varying amounts of incubation time following varying heat, radiation, and thermoradiation treatments would be very instructive. Such a study might focus on the aspect of this work showing that spore killing is highly correlated with large numbers of single strand breaks—perhaps enzyme inactivity is involved in this situation.

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Appendix A

Qiagen DNA Extraction Procedures

1. Prepare Buffers B1, B2, QBT, QC, and QF according to the following:

Buffer B1: Dissolve 18.61 g Na₂EDTA·2H₂O and 6.06 g Tris base in 800 ml distilled water. Add 50 ml 10% Tween-20 solution and 50 ml 10% Triton X-100 solution. Adjust the pH to 8.0 with HCl. Adjust the volume to 1 liter with distilled water.

Buffer B2: Dissolve 286.59 g guanidine HCl in 700 ml distilled water. Add 200 ml of 100% Tween-20. Adjust the volume to 1 liter with distilled water. pH does not need to be adjusted.

Buffer QBT: Dissolve 43.83 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol and 15 ml 10% Triton X-100 solution. Adjust the volume to 1 liter with distilled water.

Buffer QC: Dissolve 58.44 g NaCl, 10.46 g MOPS (free acid) in 800 ml distilled water. Adjust the pH to 7.0 with NaOH. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

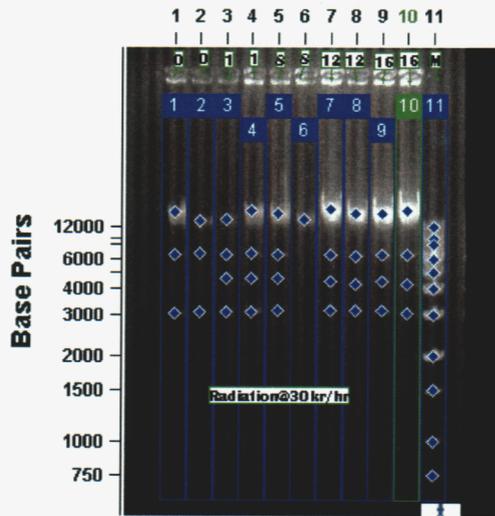
Buffer QF: Dissolve 73.05 g NaCl and 6.06 g Tris base in 800 ml distilled water. Adjust the pH to 8.5 with HCl. Add 150 ml pure isopropanol. Adjust the volume to 1 liter with distilled water.

2. For each preparation, add 2 µl of RNase A solution (100 mg/ml) to a 1 ml aliquot of Buffer B1. Dissolve lysozyme in distilled water to a concentration of 100 mg/ml.
3. Pellet bacteria from an appropriate volume of culture by centrifugation at 3000-5000 x g for 5-10 minutes. Discard the supernatant, ensuring that all liquid is completely removed.
4. Resuspend the bacteria pellet from step 3 in 1 ml of Buffer B1 (with RNase A) by vortexing at top speed.
5. Add 20 µl of lysozyme stock solution (100 mg/ml) and 45 µl of Proteinase K stock solution (20 mg/ml). Incubate at 37° C for at least 30 minutes.
6. Add 0.35 ml of Buffer B2 and mix by inverting the tube several times or by vortexing for a few seconds. Incubate at 50° C for 30 minutes.

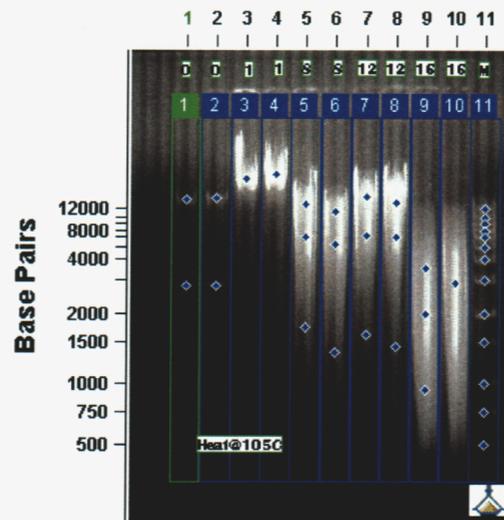
7. Equilibrate a Qiagen Genomic-tip 20/G with 1 ml of Buffer QBT and allow the Qiagen Genomic-tip to empty by gravity flow.
8. Vortex the sample supernatant for 10 seconds at maximum speed and apply it to the equilibrated Qiagen Genomic-tip. Allow it to enter the resin by gravity flow.
9. Wash the Qiagen Genomic-tip with 3 x 1-ml of Buffer QC.
10. Elute the genomic DNA with 2 x 1-ml of Buffer QF.
11. Precipitate the DNA by adding 1.4 ml room-temperature isopropanol to the eluted DNA. Recover the precipitated DNA as described in step 12.
12. Mix and centrifuge immediately at $>5000 \times g$ for at least 15 minutes at 4°C . Carefully remove the supernatant.
13. Wash the centrifuged DNA pellet with 1 ml of cold 70% ethanol. Vortex briefly and centrifuge at $>5000 \times g$ for 10 minutes at 4°C . Carefully remove the supernatant without disturbing the pellet. Air-dry for 5-10 minutes, and resuspend the DNA in 0.1-2 ml of 10 mM Tris-Cl, pH 8.5. Dissolve the DNA overnight on a shaker or at 55°C for 1-2 hours.

Appendix B

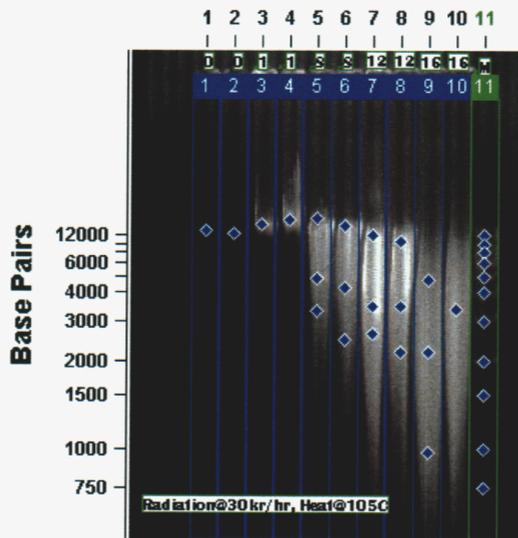
DNA Electrophoresis Gel Pictures



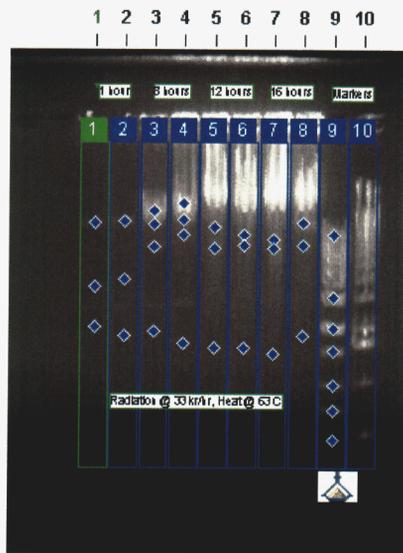
Neutral gel, radiation at 33 kr/hr.



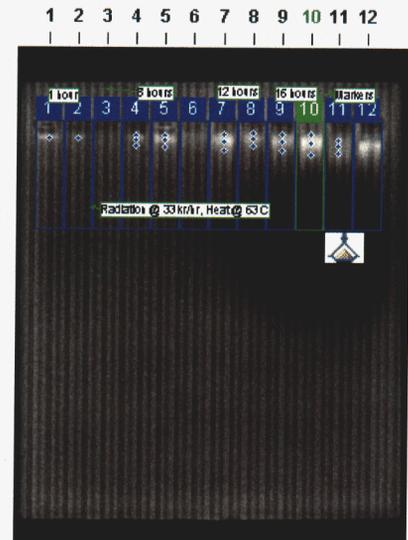
Neutral gel, heat at 105° C.



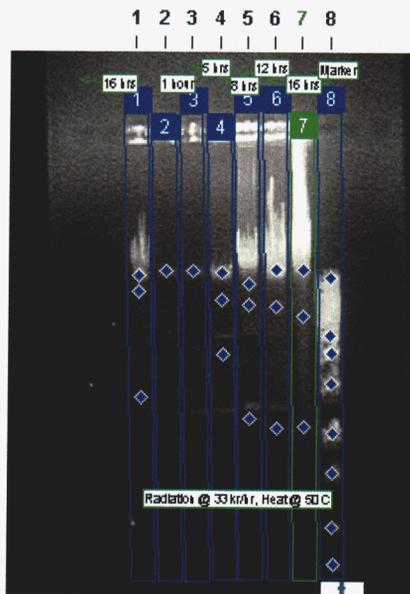
Neutral gel, radiation at 33 kr/hr, heat at 105° C.



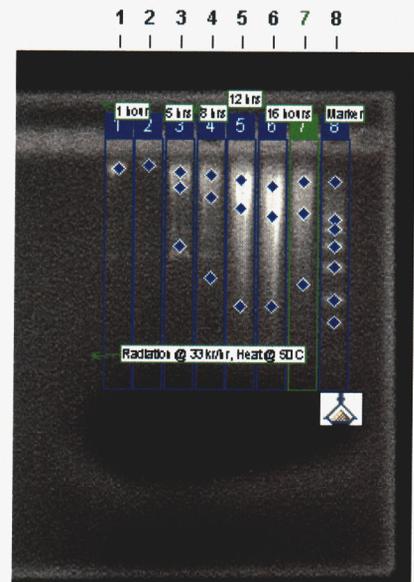
Neutral gel, 33 kr/hr, 63° C.



Alkaline gel, 33 kr/hr, 63° C.



Neutral gel, 33 kr/hr, 50° C.



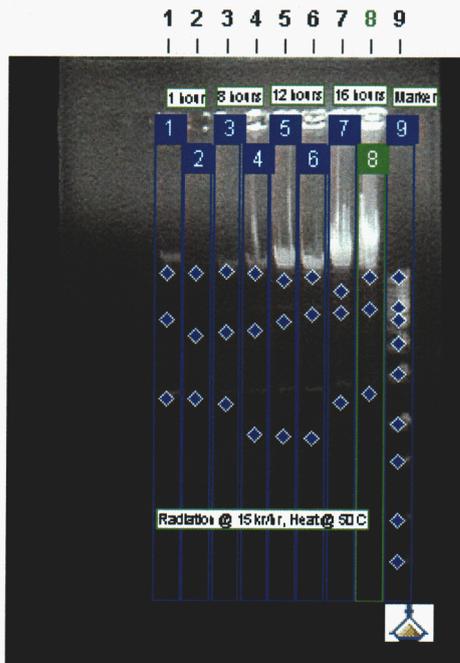
Alkaline gel, 33 kr/hr, 50° C.



Neutral gel, 50° C.



Alkaline gel, 50° C.



Neutral gel, 15 kr/hr, 50° C.



Alkaline gel, 15 kr/hr, 50° C.

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