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DEOXYRIBONUCLEIC ACID (DNA) REPAIR IN
MUSTARD-RESISTANT BACTERIA

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by Joseph Dennis
William E. White, Ph.D.

RESEARCH DIRECTORATE

April 1987



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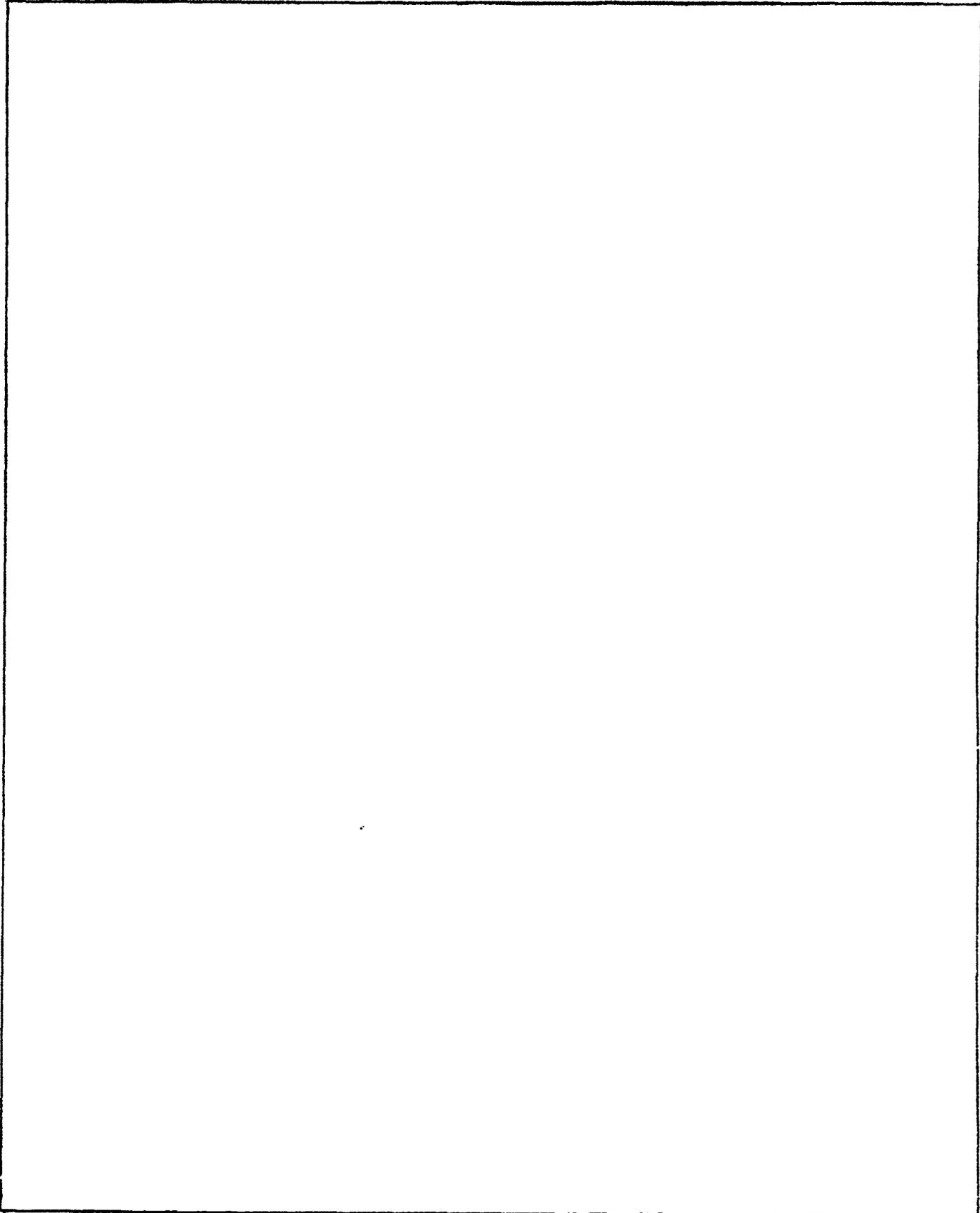
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PREFACE

The work described in this report was authorized under Project No. 1L162706A553F, CB Decontamination and Contamination Avoidance. This work was started in June 1985 and completed in August 1985.

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DEOXYRIBONUCLEIC ACID (DNA) REPAIR IN MUSTARD-RESISTANT BACTERIA

1. INTRODUCTION

Because of the caustic and corrosive nature of the solutions available for decontaminating equipment and personnel that have been exposed to chemical agents, considerable interest in developing alternatives exists. Enzymes offer many attractive features. They function efficiently at ambient temperatures under physiological conditions. Their catalytic properties should reduce much of the logistic burden imposed by DS-2 and supertropical beach which must be transported to the site and used without further dilution. Because mustard is one of the most persistent agents, an active decontamination program must be employed to clean equipment. Unlike some chemical agents, mustard will not blow away or decompose in a few minutes.

Several sources of enzymes for degrading G-type agents have been reported.¹ However, none have been identified for mustard. To discover some microbial enzymes for possible use as decontaminants, soil previously exposed to mustard was cultured with salts, sugar, and increasing concentrations of mustard. Isolated bacteria were 2 to 3 orders of magnitude more resistant than laboratory cultures of *Escherichia coli* (*E. coli*) 25922 and *Salmonella typhimurium* (*S. typhimurium*) TA1535.² Unfortunately, these bacteria did not catalyze the degradation of mustard. Aqueous solutions hydrolyzed at the same rate in the presence and absence of sonicates of bacteria.³ Also, sonicates were incubated overnight with mustard in acetone according to Zaks and Klibanov,⁴ but the mustard concentration remained constant.

Because data indicate that the bacterial resistance is not due to enzymatic degradation, the hypothesis was formulated that mustard resistance is due to an enhanced capability to repair deoxyribonucleic acid (DNA) and that the bacteria were selected from the soil on the basis of DNA repair. If this hypothesis is correct, the selected bacteria should have increased resistance to ultraviolet (UV) light which exerts its principal cytotoxicity through DNA damage.

The principal type of UV damage to DNA is the formation of pyrimidine dimers. However, other photochemical reactions such as photohydration do occur. Irradiation of an aqueous thymidine solution gives a very poor yield of thymidine dimers. However, because the bases are optimally oriented, the quantum yield for adjacent thymidines in the B form of DNA is about one. If not repaired, the thymidine dimer leads to mutations during replication when the incorrect base is inserted in the new or nascent DNA opposite the lesion. Usually, distortion in the double helix caused by the dimerization is recognized by a repair system prior to replication.

Although each type of cell contains several enzymatic systems for DNA repair,⁵ excision repair is the most common. The four steps in this repair sequence involve (1) recognition of the defect by an endonuclease and introduction of a single strand break, (2) removal of the damaged region by an exonuclease, (3) insertion of the correct bases by a DNA polymerase using the complementary strand as a template, and (4) formation of the final bond between the nascent and the original DNA by a DNA ligase.⁶

2. MATERIALS AND METHODS

Five strains of bacteria were cultured in nutrient broth. The strains included *E. coli* 25922, *S. typhimurium* TA1535, two pseudomonas strains (107-7 and 108-17), which were isolated from Carrol Island, and an untyped *Bacillus* (herein designated as B-1), which was isolated from soil near "0" field at Aberdeen Proving Ground.

For the initial phases of the experiment, culturing was done in test tubes. Latter phases necessitated large quantities of bacteria for centrifugal harvesting. These were grown in 500-ml bottles and agitated in a shaker-bath at 37 °C. All plating was done in nutrient agar.

For a UV source, a standard Gates MR-4 lamp equipped with a G8T5 8-watt germicidal bulb was used. This bulb has maximum emission at 254 nm which is the optimum for producing thymidine dimers in vitro.⁷ To obtain a constant output, the lamp was allowed to warm for 30-40 min before use. Exposure of the cultures to UV was controlled by using index cards as shutters. Eye protection from the reflected UV was absolutely mandatory. A Spectroline DIN series radiometer with digital read-out was used to measure UV intensity. Because prolonged exposure would have desensitized the unit, care had to be taken when using it.

3. RESULTS AND DISCUSSION

Initially, range finding exposures with *E. coli* 25922 and *S. typhimurium* TA1535 were done to determine an optimum intensity and working distance under the UV lamp. An exposure time of 30 sec was selected for ranges 5, 10, 15, 20, 25 and 30 cm. UV radiation ranged from 3.75×10^4 ergs/sec/cm² at 5 cm to 3.75×10^3 ergs/sec/cm² at 30 cm.

Four-hour broth cultures, showing slight turbidity, were estimated to contain a bacterial concentration of 1.0×10^8 /ml. Two milliliters were aseptically pipetted into 35x10-mm petri dishes for exposure. After exposure, serial dilutions were performed in sterile nutrient broth. Exposures of greater distance were diluted to 10^{-5} and 10^{-6} , whereas exposures of lesser distance were diluted to 10^{-3} and 10^{-4} . To provide a valid average count, five to seven plates of each dilution were made. Figure 1 shows the two survival rates obtained. We found that a 15-cm range with a 1.14×10^4 -ergs/sec/cm² intensity provides a suitable working range.

In the next phase, all five strains were exposed in random order to a 1.14×10^4 -ergs/sec/cm² intensity at a 15-cm range for 0, 5, 10, 30, 60, 90, and 120 sec. Following irradiation, the bacteria were serially diluted and plated immediately. The procedure followed was identical to that of the previous experiment; however, because they exhibited increased survival, strains 107-7, 108-17, and B-1 were also irradiated for 360 sec.

Figure 2 compares the survival rates of all five strains following irradiation. *E. coli* 25922 and *S. typhimurium* TA1535 were the most sensitive. Both showed less than 0.04% survival after 90 sec (1.03×10^6 ergs/sec/cm²). Strains 107-7 and 108-17 were the most resistant, whereas the B-1 strain was intermediate. From 240 to 360 sec, the resistant strains displayed a plateau

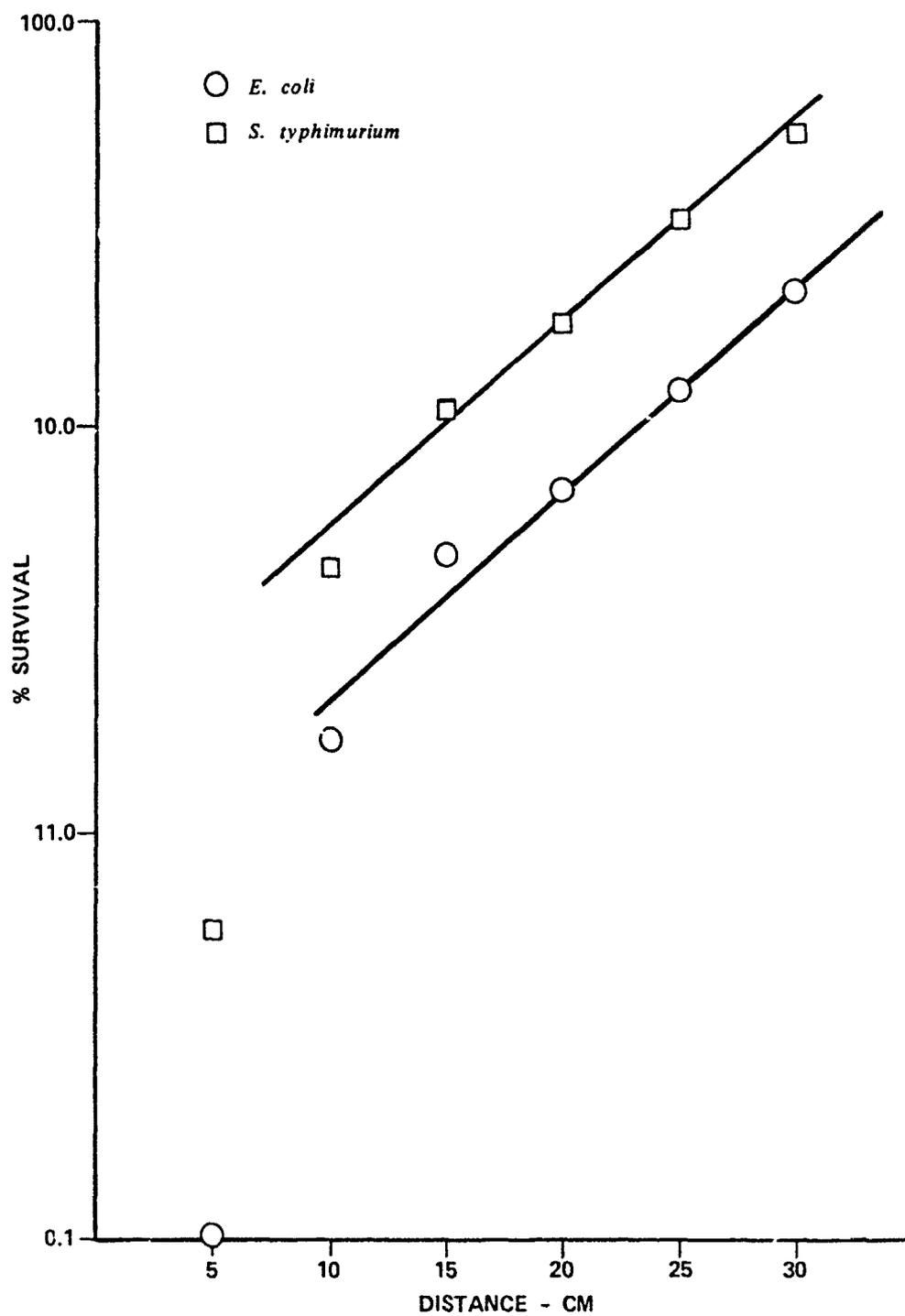


Figure 1. Effect of distance from Lamp on Cell Survival. *E. coli* 25922 and *S. typhimurium* TA1535 were grown and photolyzed for 30 sec at the indicated distance, diluted, and plated on nutrient broth. Resulting colonies were counted after 20 hr.

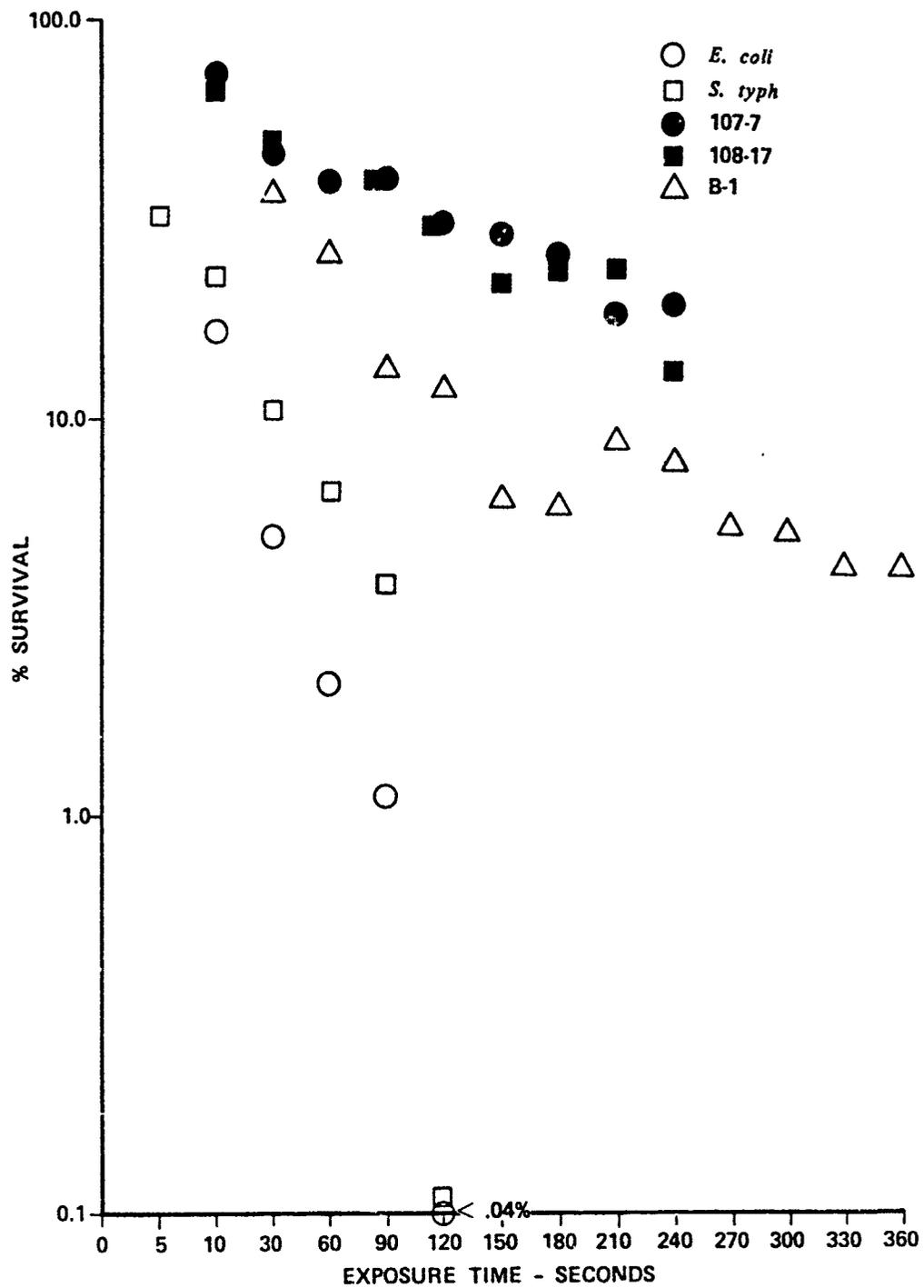


Figure 2. Effect of Time of Photolysis on Survival of Selected Bacteria. Each strain was grown overnight in nutrient broth, diluted, photolyzed for the indicated duration at 1.1×10^4 $\mu\text{s}/\text{sec}/\text{cm}^2$ (15 cm) in nutrient broth, diluted, plated immediately on nutrient broth, and counted the following day.

in the survival curve. This plateau did not result from the selection of a subpopulation with enhanced UV resistance because an identical survival curve was obtained with a culture grown from one of the "plateau" colonies.

Previous experiments determined the bacteria's relative resistance to UV. The next phase involved irradiating the bacteria in a liquid-holding medium that allowed excision repair of DNA but prohibited cell division due to a lack of nutrients.⁸ The bacteria contained sufficient energy and intermediates to repair damaged DNA but not enough to totally replicate DNA, thereby initiating cell division.⁹ Thus, there was time to repair the DNA before replication "set" the damage and introduced a lethal mutation.

Selecting a suitable liquid-holding medium, which would preserve viability, was the first step. Bacteria were grown overnight and centrifuged at 2500 rpm (1500xg) for 30 min at 4 °C. After decanting the supernatant, 5 ml of phosphate buffered saline (PBS) was added to each tube, which was then vortexed and centrifuged for 30 min.

Initial platings indicated that very few colonies survived even in the absence of UV irradiation. When the PBS wash was eliminated, the colony count increased dramatically; however, after 50 min of refrigeration, viability decreased. Normal saline and demineralized water were tried in place of PBS; the saline and water also resulted in a more rapid decrease of viable cells (Figure 3). Eventually, a 50-mm solution of monobasic phosphate buffer, adjusted to pH 7 and filter sterilized, proving the buffer to be an excellent liquid-holding medium for all five strains.

Each bacterial strain was suspended and diluted with phosphate buffer following harvesting. A series of 2-ml aliquots was added to 35x10 mm petri dishes that were irradiated with UV light of 1.14×10^4 ergs/sec/cm² for 5, 10, and 30 sec, respectively. Following exposure, all laboratory manipulations were performed in dim light to minimize the effects of photoactivation. Immediately after irradiation, a 0.1-ml aliquot was plated as a zero time control. The remaining bacterial suspensions were refrigerated until plating at 5-, 30-, and 60-min intervals.

Figure 4 illustrates the survival rates for *E. coli* 25922 and *S. typhimurium* TA1535. Although both are sensitive to UV if plated immediately, the survival of *E. coli* 25922 but not the survival of *S. typhimurium* TA1535 increases during liquid holding. *E. coli* 25922 has intact excision repair, whereas *S. typhimurium* TA1535 (one of the Ames strains for mutation testing) has defective excision repair.

Figure 5 shows UV survival in a liquid-holding medium for pseudomonas strains 107-7 and 108-17. Both are somewhat equally resistant to UV damage and display proficiency for excision repair. Strain 108-17 displayed greater excision repair than the 107-7 strain.

Figure 6 shows the survival of B-1 following irradiation and liquid holding. Although B-1 has the greatest resistance to UV when irradiated in phosphate, there was no increase in survival during liquid holding. It is possible that the repair was too rapid to be measured by this technique, DNA was repaired by another mechanism, or the resistance was due to some other factor.

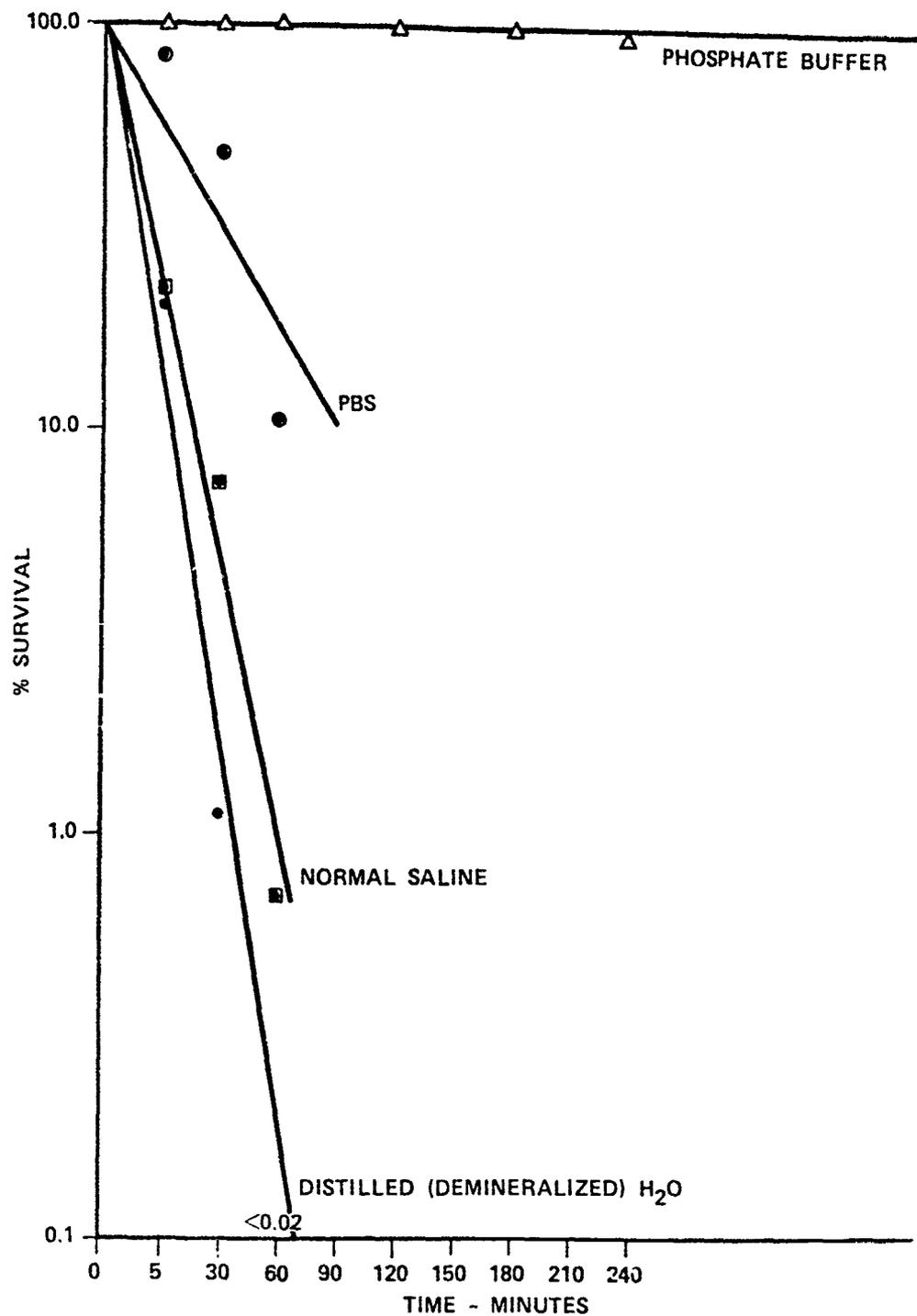


Figure 3. Development of Liquid-Holding Solutions. Effect of different buffers on survival of *S. typhimurium* TA1535. Bacteria were grown, harvested, and suspended in buffer for the indicated period, plated, and counted the following day.

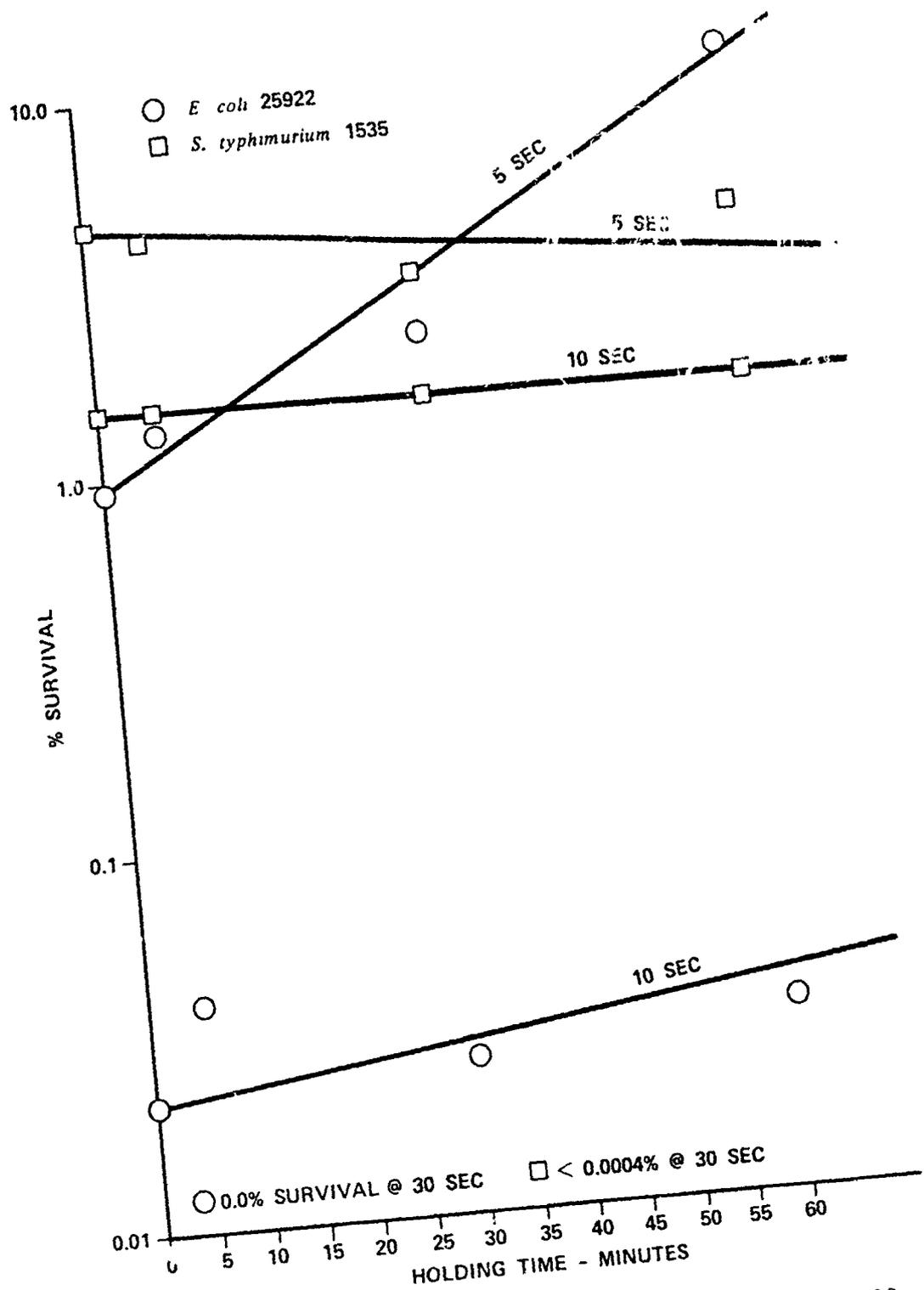


Figure 4. Effect of Liquid Holding on Survival. *E. coli* 25922 and *S. typhimurium* TA1535 were grown in nutrient broth, harvested, suspended in phosphate buffer, photolyzed for 5 or 10 sec, and held for the indicated period before plating. Resulting colonies were scored the following day.

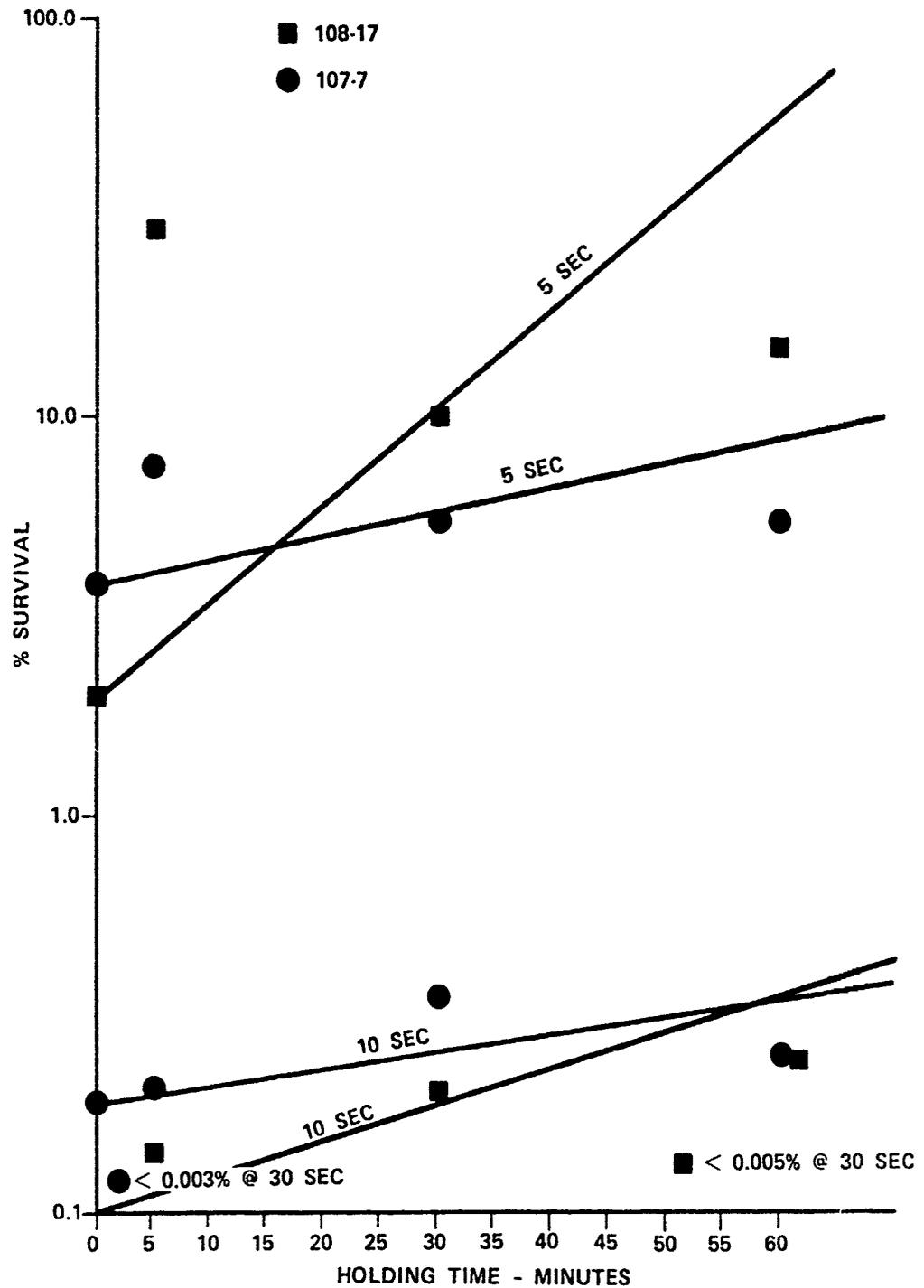


Figure 5. Effect of Liquid Holding on the Survival of Mustard-Resistant Bacteria 107-7 and 108-17. Bacteria were grown in nutrient broth, harvested by centrifugation, suspended in phosphate buffer, photolyzed for 5 or 10 sec, and held at room temperature for the indicated interval before plating.

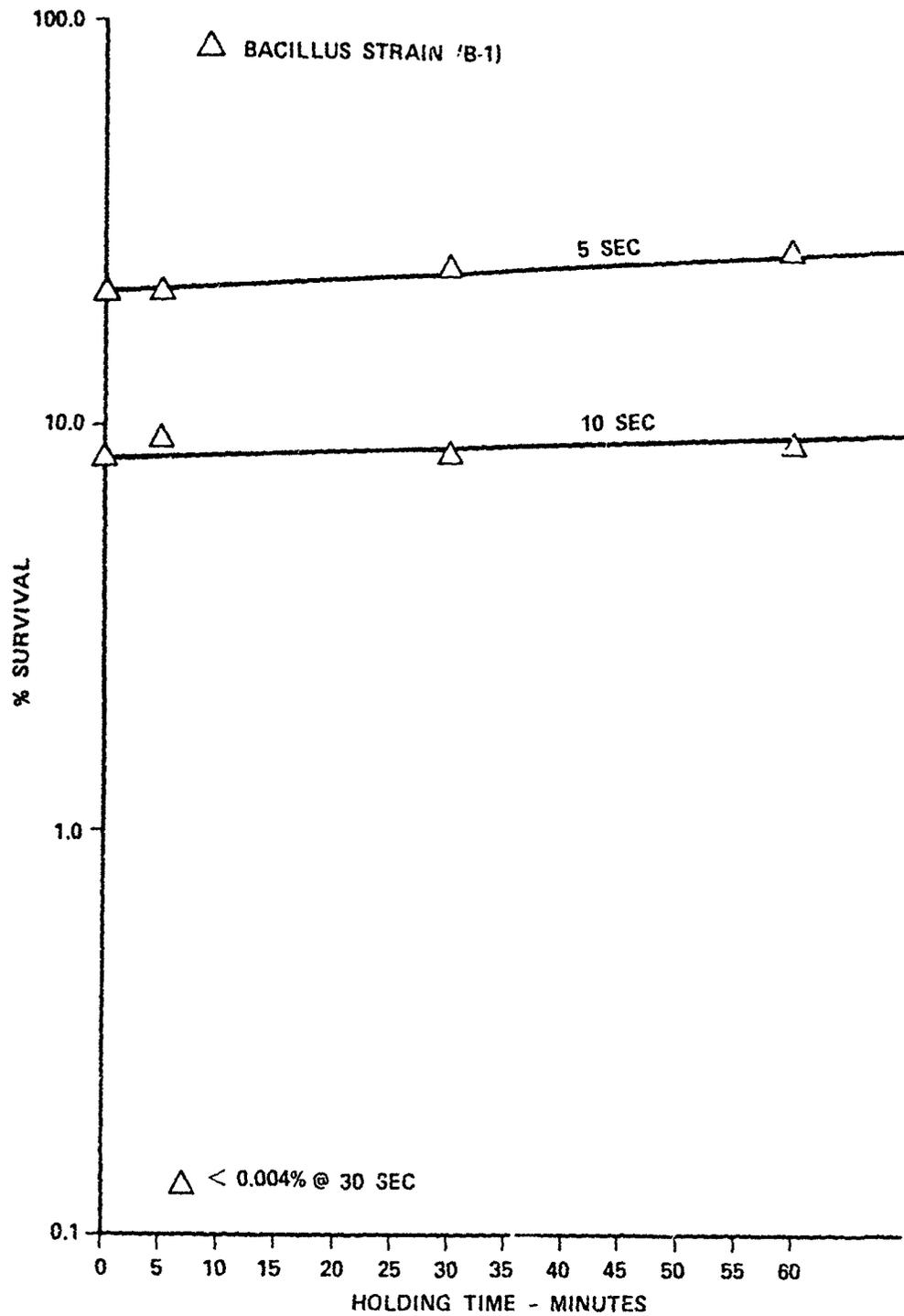


Figure 6. Effect of Liquid Holding on the Survival of Mustard-Resistant Bacteria B-1 following Photolysis for 5 or 10 sec. Bacteria were grown in nutrient broth, harvested by centrifugation, suspended in phosphate buffer, irradiated, and held at room temperature for the indicated interval before plating.

4. CONCLUSIONS

The strains that were resistant to mustard were also resistant to UV irradiation. The survival of E. coli 25922, which has intact excision repair, but not S. typhimurium TA1535, which has deficient repair, increased during liquid holding. Resistant strains 108-17 and 107-7 also showed increased survival during liquid holding.

This data supports, but does not conclusively prove, the hypothesis that the resistance of the bacteria to mustard results from an enhanced DNA repair capability.

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