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# THE CONTRIBUTION OF ENDOGENOUS AND EXOGENOUS DAMAGE TO THE TOTAL RADIATION-INDUCED DAMAGE IN THE BACTERIAL SPORE.

PROGRESS REPORT

G.P. Jacobs*, A. Samuni** and G. Czapski***

* Department of Pharmacy

** Department of Molecular Biology

*** Department of Physical Chemistry

The Hebrew University, Jerusalem, Israel.

Work carried out during the period:

December 1st, 1979 to November 30th, 1980.

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# The Contribution of Endogenous and Exogenous Damage to the Total Radiation-Induced Damage in the Bacterial Spore.

G.P. JACOBS*, A. SAMUNI†, and G. CZAPSKI‡

* Department of Pharmacy, School of Pharmacy

† Department of Molecular Biology

‡ Department of Physical Chemistry,

Hebrew University of Jerusalem, Jerusalem, Israel.

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All correspondence to: Dr.G.P. Jacobs,
Department of Pharmacy, School of Pharmacy,
Hebrew University, P.O.B. 12065
Jerusalem, Israel.

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Abstract

Radical scavengers such as polyethylene glycol 4000 and bovine albumin have been used to define the contribution of exogenous and endogenous damage to the total radiation-induced damage in aqueous buffered suspensions of Bacillus pumilus spores. The results indicate that this damage in the bacterial spore is predominantly endogenous.

Key Words: bacterial spores; γ-irradiation; Bacillus pumilus; exogenous damage; endogenous damage.

## INTRODUCTION

As a prerequisite to the identification of those radical species responsible for the indirect portion of the radiation-induced damage in different biological test-systems, it has been considered useful to determine whether the origin of this damage is endogenous or exogeneous to the damaged cell. Several reports (1-4) ascribed part of the biological damage, especially that due to $O_2$, to radicals originating outside the cell. It has even been suggested that superoxide radicals originating exogenously might be responsible for endogenous cellular damage (2-5). Since it seems highly unlikely that reactive water radicals, produced in the suspending medium will penetrate into the spore, a knowledge of the type of damage would help determine whether the damaging species originate within the cell matrix or in the surrounding milieux.

There is some disagreement in the earlier literature (6, 7 ) as to whether, for example, irradiation of phage in dilute buffered suspensions primarily affects the viral proteins or the DNA. A recent report from our laboratories ( 8 ) on the $\gamma$-irradiation of T4 bacteriophage suspended in phosphate buffer containing appropriate radical scavengers, conclusively showed that roughly 90% of the damage stems from the water radicals generated in the bulk suspension, which probably affected phage proteins, whilst the remaining small portion of the radiation damage is initiated inside the phage and involves the viral DNA.

More recently, in a study on the roles of superoxide radicals and molecular oxygen in radiation-induced damage of E.coli B suspended in dilute phosphate buffer ( 9 ), it has been shown that damage is almost exclusively endogenous, that is, its origins are intracellular, a finding in contradiction to that observed by other investigators ( 1, 3, 4 ).

The relative contributions of the endogenous and exogenous radiation effects might be determined by the volume-to-surface ratio of the biological target. Since in spores, because of their size, the ratio is smaller than in the vegetative cell, a higher contribution for the exogenous effect might be anticipated.

It has generally been assumed that the damaging species in spores irradiated in aqueous ( and non-aqueous ) suspensions originate intracellularly ( 10, 11 ). For example, Al-Shaickly and Tallentire ( 12 ) have shown that radiation-induced $O_2$-dependent damage is associated with the spore DNA, located in the protoplast ( 13 ).

The present study, preliminary to identifying those radical species responsible for radiation induced damage in the hydrated spore, is concerned, by use of suitable radical scavengers, with defining the contribution of endogenous and exogeneous damage to the total radiation-induced damage in aqueous suspensions of spores of Bacillus pumilus E601.

## MATERIALS and METHODS

Polyethylene glycol (PEG) 4000 was supplied by Fluka and was of practical grade. Bovine albumin ( essentially fatty acid free ) was obtained from Sigma. These agents were used without further purification.

The test organism was spores of Bacillus pumilus E601 ( ATCC 27142 ) kindly supplied by Dr. Herbert Prince of the Gibraltar Biological Laboratories, U.S.A., as " test-strips " each impregnated with approximately $10^6$ spores. A test strip was placed in 10 ml of nutrient broth ( Difco ) and incubated overnight at $37^oC$. Sporulation of the resultant suspension of vegetative cells was carried out by the methodology of Powers, Ehret and Bannon ( 14 ). The spore suspension thus obtained was washed 3x in M/15 phosphate buffer ( pH 7.0 ) and held as stock at $4^oC$. It contained approximately $3 \times 10^7$ spores $ml^{-1}$. Prior to irradiation this suspension was suitably diluted in buffer solutions, containing, where necessary, PEG or bovine albumin to a concentration of $10^5$ spores $ml^{-1}$. Initial concentrations of PEG or bovine albumin were 10% and 1% respectively. Routinely spores were in contact with the additive for one hour prior to irradiation.

Irradiation was carried out at room temperature using a model M38-3 Gammator 2400 Ci $^{137}Cs$ $\gamma$ radiation source ( Radiation Machinery Corporation, U.S.A.). The average dose rate was 1.75 krads $min^{-1}$ checked by periodic dosimetric determinations using a ferrous sulphate dosimeter ($G_{Fe^{3+}} = 15.3$ ( 15 ) ). Irradiation vessels were glass

vials of od 19.7 mm sealed with gas-tight rubber closures. Arrangement of these vessels within the irradiation chamber permitted the simultaneous irradiation of 18 spore suspensions at similar dose rates. Deoxygenation was by bubbling $O_2$ - free He ( < 2 ppm $O_2$, Matheson ) for 20 minutes through the spore suspension immediately prior to irradiation. For maintenance of suspensions under aerated conditions, $O_2$ ( extra dry grade, Matheson ) was bubbled through the suspensions for 5 minutes.

Suspensions were irradiated for fixed time intervals such that at the maximum dose level at least two decades of inactivation were generally achieved. Following irradiation suspensions were appropriately diluted in double distilled water and four 0.05 ml aliquots of each diluted suspension pipetted onto plates of nutrient agar ( Difco ) containing 0.5% glucose. Following overnight incubation at 32°C, the micro colonies formed, were scored. Each colony was taken as indicative of a single spore in the original suspension. Counts performed on unirradiated samples of test suspensions treated identically to those exposed to gamma-irradiation were used as control estimates of the number of viable spores in calculations of surviving fractions.

Dose - ln survival curves were constructed from six experimental points. Curves joining these points were linear and are described by the expression:

$$\ln S = \ln n - kD,$$

where $S$ is the fraction of spores surviving a radiation dose $D$, and $k$ and $n$ are constants. Variation in values of $k$, the inactivation

rate constant, ( 16 ) with changes in conditions of test have been used to demonstrate quantitatively changes in radiation sensitivity. Values of k, together with associated standard errors, were computed by a method of least squares analysis.

## RESULTS

Typical dose survival curves for B. pumilus spore suspensions irradiated in the presence and absence of $O_2$ are depicted in Figure 1. These curves are exponential over at least two decades of inactivation. Comparison of values of inactivation constants ( Table 1 ) yield an oxygen enhancement ratio of 2.5.

In order to estimate the contribution of exogenous and endogenous radiation damage to the total damage, the influence of PEG 4000 on spore survival has been tested. This polymer ( MW$\sim$4000 is effective at scavenging exogenous $^{\bullet}OH$ radicals ( $k_{^{\bullet}OH} = \sim 7 \times 10^8\ M^{-1}s^{-1}$ ( 17 )), but is not thought to penetrate the spore. PEG 4000 at a 10% concentration in irradiated spore suspensions, is capable of reducing the basic anoxic spore response by about 8% and the oxic response by 17% ( Figure 1 ). In contrast, bovine albumin (1%) ( $k_{^{\bullet}OH} = \sim 10^{10}\ M^{-1}s^{-1}$ ( 18 )) in irradiated spore suspensions does not appear to modify the basic radiation responses ( Figure 1 ).

## DISCUSSION

Values of k for B. pumilus spore suspensions irradiated in the presence of He and $O_2$ ( 5.1 ± 0.2 and 12.6 ± 0.5 $Mrad^{-1}$, respectively ) compare favourably to values obtained by other investigators. A value of 5.8 $Mrad^{-1}$ has been reported for dried spores irradiated in anoxia and then given $H_2O$ treatment ( 19 ), whilst a value of 13.7 $Mrad^{-1}$ has been obtained for irradiated oxygenated aqueous suspensions ( 20 ).

Our original choice of PEG 4000 as a radical scavenger was based on the assumption that it cannot penetrate the spore coat and therefore the lack of modification of the basic oxic and anoxic spore responses would be confirmation of the generally accepted notion that radiation damage in the spore is endogenous. Such an argument has been put forward by Samuni et al.( 9 ) to explain the lack of modification in E. coli B by this additive. Our findings that PEG 4000 modifies the radiation response in the spore under both test conditions suggest that damage is either exogenous or that this additive can penetrate the spore. A study by Gerhardt and Black ( 21 ) however showed that PEG 4000 is in fact capable of penetrating spores of Bacillus cereus. On the other hand, a compound shown by that group to be incapable of penetrating the spore is bovine albumin, hence its use in the present study. We therefore proceeded with the assumption that modification of radiation sensitivity by bovine albumin would be indicative of exogenous damage. The lack of modification by bovine albumin that has been observed, coupled with our findings with PEG 4000, is further evidence for the notion that damage in spores of Bacillus pumilus , and spores in general, is endogenous.

The fact that the OER was not altered by bovine albumin, indicates that the radiosensitization brought about in the presence of oxygen originates inside the cell. This conclusion is in accord with, although not necessarily derived from, the lack of any exogenous radiation effect in anoxia.

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TABLE I

Effect of different irradiation conditions on response of *Bacillus pumilus* spores suspended in phosphate buffer ( pH 7.0 )

| Equilibrating gas | Additive | k ( $Mrad^{-1}$) | s.e. | Enhancement ratio[a] |
|---|---|---|---|---|
| He | | 5.1 | 0.2 | 1.00 |
| He | PEG 4000 10% | 4.7 | 0.4 | 0.92 |
| He | bovine albumin 1% | 5.3 | 0.3 | 1.04 |
| $O_2$ | | 12.6 | 0.5 | 2.47 |
| $O_2$ | PEG 4000 10% | 10.4 | 0.6 | 2.04 |
| $O_2$ | bovine albumin 1% | 12.7 | 0.4 | 2.49 |

a compared to He

Fig. 1. Effect of bovine albumin (1%) and PEG 4000 (10%) on the radiosensitivity of buffered suspensions ( pH 7.0 ) of Bacillus pumilus spores.

( ● ) deaerated suspension without additive,

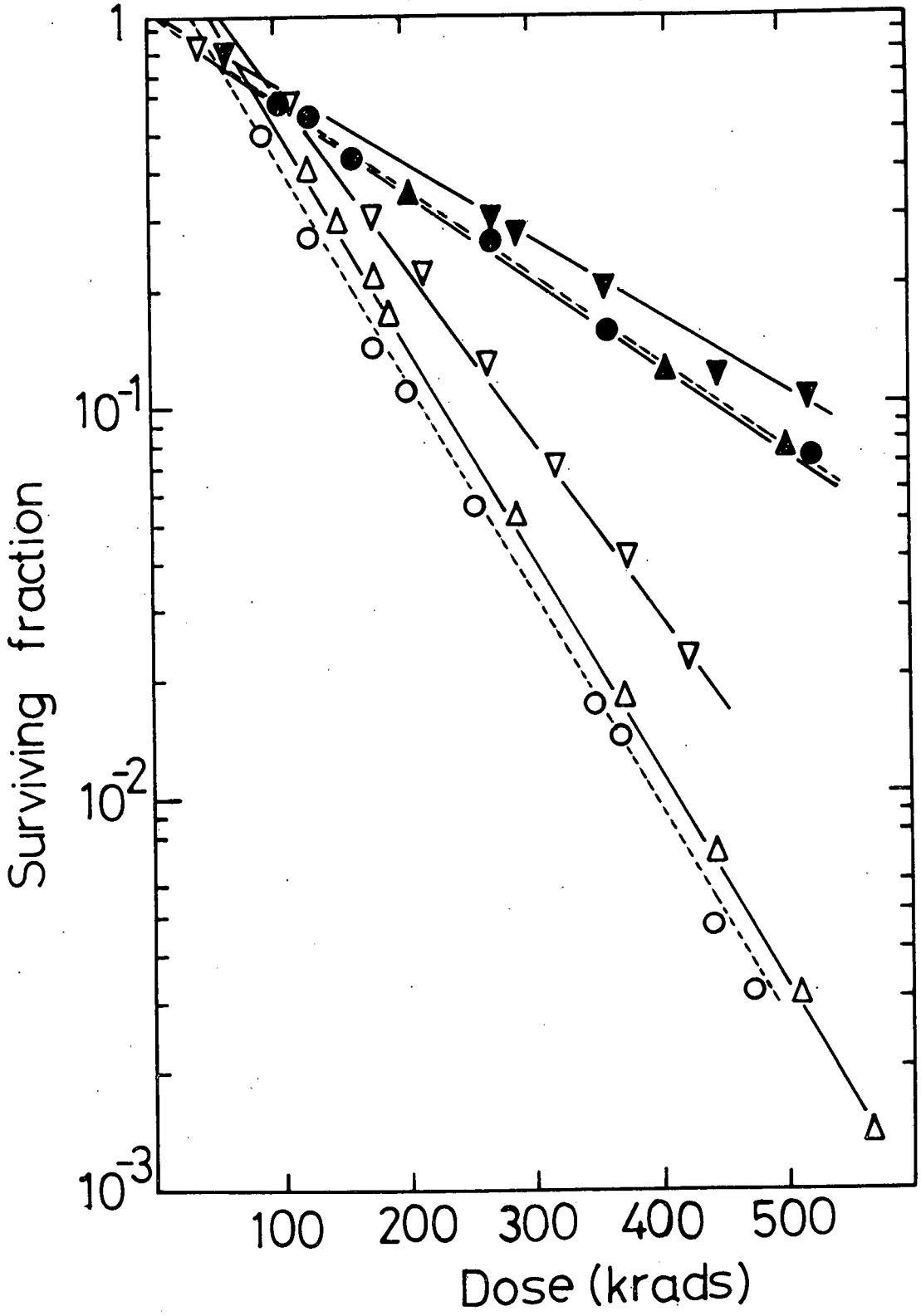
( ▼ ) deaerated suspension with PEG 4000,

( ▲ ) deaerated suspension with bovine albumin,

( o ) oxygenated suspension without additive,

( ∇ ) oxygenated suspension with PEG 4000,

( △ ) oxygenated suspension with bovine albumin


1
$10^{-1}$
$10^{-2}$
$10^{-3}$
Surviving fraction
100
200
300
400
500
Dose (krads)