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RADIATION-INDUCED DAMAGE IN T4 BACTERIOPHAGE
THE EFFECT OF SUPEROXIDE RADICALS AND MOLECULAR OXYGEN

PROGRESS REPORT

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Yael A. Ilan† and Gidon Czapski†

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Work carried out during the period:

December 1st 1977 to November 30th 1978

Prepared for the U.S. Energy Research & Development Administration
Under Contract No. EY-76-C-02-3221

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copies: 3
pages: 16, including 1 table
figures: 2

Running Title: γ-Irradiated T4: Effects of O_2 and $\dot{O}_2^-$.

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Abstract

The sensitivity of T4 bacteriophage towards γ-irradiation has been studied in phosphate buffer suspensions. The spectrum of the water radicals was controlled by a careful choice of the appropriate saturating gas and the addition of radical scavengers. Thus, it was possible to distinguish between the effects of molecular oxygen and the superoxide radicals formed through its reactions.

About 90% of the damage was caused by the water radicals formed in the bulk suspensions. These probably affected the phage proteins; only the remainder of the damage involved the viral DNA.

The oxygen enhancement ratio observed was not connected in any way with the formation of the superoxide radicals.

The results confirmed that the ÔH radicals are the reactive species, while e^-_{aq} as well as the superoxide radical do not contribute to the radiodamage.

Samuni A., Chevion M., Halpern Y.S., Ilan Y.A. and Czapski G., Radiation-Induced Damage in T4 Bacteriophage: The Effect of Superoxide Radicals and Molecular Oxygen. *Radiat. Res.*

key words: superoxide radicals; γ-irradiation; oxygen effect; T4 bacteriophage.

Introduction

The sensitivity of biological systems towards ionizing radiation is generally higher in the presence of oxygen than when they are irradiated under anoxic conditions. Although this phenomenon has been extensively investigated and discussed, there is no satisfactory interpretation for the oxygen effect (1-2). This effect is often expressed as oxygen enhancement ratio (OER) which is defined as the quotient of the aerobic to anaerobic radiation-sensitivity. With bacterial cells, it was found that molecular oxygen enhances the radiation-induced lethality. In the case of viruses, on the other hand, this oxygen effect was often reported to be negligible (3-5).

Several explanations were suggested to account for the differences between radiodamage in oxic and anoxic conditions. These included: rapid repair mechanisms preferentially active in anoxia (5-8); changes in the level of endogenous sulfhydryls serving as radioprotectants (9-10); direct interaction of molecular oxygen with the target biomolecules (11); attack by superoxide radicals (12); secondary production of $\dot{O}H$ radicals formed through the Haber-Weiss reaction (13) ($\dot{O}_2^- + H_2O_2 \rightarrow OH^- + \dot{O}H + O_2$) which enhances the radiodamage (14).

In contrast with bacterial cells where repair mechanisms may continuously function, the radiation-induced damage in viruses remains unmodified. In these organisms, an enhancement of the damage by oxygen was observed when they were irradiated either within the host cell (15) or in the presence of radioprotectants (5-6, 10, 16).

In dilute solutions the ionizing radiation interacts primarily with the solvent, thus yielding the primary water radicals $\dot{H}$, $\dot{O}H$ and e^-_{aq}. These free radicals react in turn with the solubilized biomolecules, and are thus re-

sponsible for the *indirect* radiation damage. In addition, a fraction of the radiation energy brings about a direct excitation of the biomolecules and is thus responsible for the *direct* radiation damage (11). The biomolecules then may undergo further chemical modifications. Oxygen, if present, can react with the target biomolecules and thus prevent any possible restitution or repair processes. Also, through reaction with oxygen, the free radicals $\dot{H}$ and e^-_{aq} are converted into superoxide radicals which are relatively less active. Usually both the *direct* and *indirect* routes for radiation damage occur simultaneously. Since oxygen affects each of them in a different way, the overall role of oxygen in radiodamage is obscure.

In the present study, we concentrate on the indirect effect of γ-radiation on the lethality of T4 bacteriophage in dilute suspensions. Special attention is paid to the elucidation of the role of superoxide radicals and of oxygen in this system.

Materials and Methods

All chemicals used were of the highest analytical grade available and were used without further purification. Bacto-Agar and Nutrient Broth were obtained from Difco; Sodium formate and Sodium chloride from B.D.H; polyethylene glycol (PEG), practical grade, was purchased from Fluka. All the solutions were made up from triply-distilled water. The phage used was T4 and the bacterial strain was Escherichia coli B. Superoxide dismutase (SOD) was a product of Sigma isolated from bovine blood (2000 units/mg protein). Heat-inactivated enzyme was prepared by boiling SOD for 30 min. The bacteriophages were γ-irradiated in sealed glass bottles ($\sim$ 9ml) within a ^{137}Cs gamma source (M Gamma Meter Radiation Machinery Co. Model M38/3, dose-rate 96 Krad $\cdot g^{-1} \cdot h^{-1}$). Irradiations of the bacteriophage suspensions ($\sim 4 \cdot 10^5$ PFU$\cdot ml^{-1}$) were carried out at room temperature in phosphate buffer 0.05M, pH 7.0. The fraction of bacteriophage that survived the exposure was appropriately diluted, then preadsorbed to exponentially growing bacteria at a multiplicity <0.01, and plated in triplicates. The dilutions were such as to give 50-200 plaques per plate. The plates were incubated at 37° overnight and the plaques were counted. The radiation dose was measured using a Fricke dosimeter (12).

Deoxygenation, when required, was carried out by bubbling either argon or nitrous-oxide through the bacteriophage suspensions prior to irradiation. To ensure complete removal of the oxygen, the displacing gas was first passed through a series of oxygen-traps (V^{2+}, HCl, Hg/Zn). In experiments where the presence of both O_2 and N_2O was desired, the N_2O-saturated phage suspensions were appropriately diluted with air-saturated phage suspension. Control experiments demonstrated that the bubbling by itself (not followed by radiation) did not lower the titer of the phage.

Irradiations were carried out at room temperature.

Results and Discussion

Deaerated and aerated suspensions of T4 bacteriophage ($\sim 4 \cdot 10^5$ PFU·ml^{-1}) were γ-irradiated within a ^{137}Cs Gamma source, and the fractions of bacteriophage that survived various irradiation doses were determined. The survival data are represented as semilogarithmic dose-response plots in Fig. 1 (traces a and b). Both traces exhibit the typical "shoulder" which generally characterizes "multi-hit" dose-response curves (11), and within the range of that "shoulder" they look practically the same. Beyond a certain dose, however, the survival curves approach an exponential dependence with a steeper slope observed for the aerated system. The $D_{37\%}$ values were calculated from these slopes and are presented in Table I. Our experiments were carried out in dilute buffer solutions; the oxygen effect seen in Fig. 1 is in accordance with the general observation for OER in biological systems. Nevertheless, in dilute solutions where the *indirect* radiation effect predominates, little or no oxygen enhancement was observed in many cases (2-5). In fact, under such conditions where the damage was mainly caused by the water radicals, protective effects exerted by oxygen were even observed in some cases (17). In oxygenated (or air-saturated) solutions, practically all e^-_{aq} and $\dot{H}$ radicals are scavenged by the molecular oxygen to yield the $\dot{O}_2^-$ radicals. Thus, a possible explanation for the oxygen effect could be the implication of the superoxide radicals as responsible for the extra damage. This indeed has been previously suggested by Misra and Fridovich (12, 14). To examine this assumption in our system the T4 bacteriophages were irradiated in oxygenated suspension containing 0.01 M formate ions. Under these experimental conditions all the water radicals were converted into superoxide radicals. The results are presented as a dose-response survival curve in Fig. 1c. The trace displays an exponential

dependence on dose, which fits a "single-hit" curve without a "shoulder", with $D_{37\%}$ = 32 Krad. This result indicates that with superoxide radicals the radiation-sensitivity of the bacteriophage was considerably reduced and the nature of the damage appeared different.

The effect of superoxide radicals

In the presence of O_2 and formate, the $D_{37\%}$ for the phages increased almost ten-fold (see Table I). This implies that the superoxide radicals are (as is now generally accepted) much less harmful to the bacteriophage than the $\dot{O}H$ radicals. Yet the protection effect thus obtained was only partial, indicating that the $\dot{O}_2^-$ radicals are possibly not completely inactive. In order to distinguish this possibility from the damage due to the *direct* radiation effect, the influence of the addition of superoxide dismutase (SOD) was examined. The irradiation was carried out in oxygenated suspensions containing 0.01 M formate and 20 $\mu g \cdot ml^{-1}$ SOD. In the control experiments the added SOD had been previously heat-inactivated. Here all the water radicals generated in the bulk suspension, outside the virus, were converted into $\dot{O}_2^-$ radicals which in turn were removed from the system by the SOD enzyme (3, 14). The introduction of the enzyme did not show any effect on the survival-curves of the phage (see Fig. 1c). Further evidence for the above conclusion was obtained when the T4 phages were irradiated in the presence of polyethylene glycol (PEG). This polymeric alcohol (M.W. $\sim$ 2000), which does not penetrate the virus, efficiently scavenges the $\dot{O}H$ radicals formed in the bulk suspension (18). Fig. 2c shows the dose-respone curve obtained when the phages were irradiated in an N_2O saturated suspension containing 0.05 M PEG. This trace exhibits an exponential dependence on dose and does not differ from the curve observed

in the presence of oxygen and formate ions, where only superoxide radicals are present (Fig. 1c). Furthermore, the $D_{37\%}$ values derived for both cases are practically the same. These experiments confirm the conclusion that the exogenous $\dot{O}_2^-$ does not damage the phage, while the inactivation seen in Fig. 1c is due to *direct* radiation effects and water radicals formed inside the phage.

Damage due to *exogenous* water radicals

The marked protection provided by the PEG shows that in dilute buffer solutions in the absence of radical scavengers, the damage stems primarily from the water radicals generated in the bulk suspension. A small portion of the radiodamage is initiated inside the phage. Thus the distinction made here is not between *indirect* and *direct* radiodamage, but rather between the *exogenous* and the endogenous *effects*. The different nature of these two effects is clearly manifested by the difference in shape of the respective dose-response curves. In experiments where the *exogenous* damage predominates, it seems highly unlikely that the reactive water radicals will penetrate the phage-coat and hit the viral DNA inside. Indeed, the present results, which show dose-response curves with "multi-hit" character, are in good agreement with previous suggestions that phage-irradiation in dilute buffered suspensions primarily affects the viral proteins (19). In comparison, "single-hit" curves seen in the case of *endogenous* damage agree with the suggestion that in the presence of radioprotectants the DNA is damaged (19). This conclusion does not support reports that even in dilute suspension it is the DNA which is affected (20).

Effect of H_2O_2

Recently it was suggested (14) that secondary ÔH radicals which are produced through the Haber-Weiss reaction (13) from H_2O_2 and superoxide radicals cause further damage during irradiations. According to this explanation, the combined presence of H_2O_2 and $\dot{O}_2^-$ may contribute to the OER. To examine this assumption, we irradiated aerated and deaerated suspensions of T4 phage in the presence of 0.001 M hydrogen peroxide. In both cases no effect of H_2O_2 on the radiosensitivity was observed. These results support the ruling out of the Haber-Weiss reaction in these systems on the grounds of kinetic considerations, as was previously suggested (21).

Effect of molecular oxygen

Both traces a and b in Fig. 1 appear as "multi-hit" curves with an initial low sensitivity to radiation, followed by an approximately exponential portion with higher radiosensitivity. The two curves differ in their exponential parts. Comparison of the $D_{37\%}$ values for the two systems shows an apparent OER of about two (see Table 1). Certainly, this oxygen effect does not stem from the conversion of $\dot{H}$ and e^-_{aq} into $\dot{O}_2^-$, since the superoxide radicals were shown not to contribute to phage inactivation. Phage inactivation and the oxygen effect were also studied in N_2O saturated suspensions, in the absence and in the presence of oxygen. The results are summarized in Fig. 2, traces a and b. In oxygen-free solutions in the presence of N_2O only, the e^-_{aq} radicals are scavenged by the nitrous oxide and converted into ÔH radicals, the concentration of which is practically doubled (see Table I). As one can see in Fig. 2a, the radiosensitivity was roughly doubled compared with an argon-saturated system (Fig. 1a). This indicates that the radiodamage was due to the ÔH radicals, while the contribution of the hydrated electrons

is negligible.

Fig. 2b shows the survival curve obtained after irradiation of the phage suspension saturated with N_2O + air (1:1 mixture). In this case, in spite of the presence of oxygen the e^-_{aq} radicals react with the nitrous oxide ($[N_2O] = 10^{-2}$ M) rather than with the oxygen ($[O_2] = 10^{-4}$ M), as $k_{O_2+e^-_{aq}} \cdot [O_2] / k_{N_2O+e^-_{aq}} \cdot [N_2O] = 0.1$. In such a way, the effect of molecular oxygen can be distinguished and examined separately from the effect of the superoxide radicals.

Traces a and b in Fig. 2 are also typical of "multi-hit" dose-response curves. At the range of the "shoulder" they do not differ from each other or from curves a and b in Fig. 1. The difference is reflected in the linear portions of the traces. Their "shoulders" are followed by an apparent exponential dependence on dose, from which the respective $D_{37\%}$ values were derived. Comparison of these values (Table I) shows that oxygen has actually doubled the radiosensitivity of the phage, in the absence of N_2O (Figs. 1a, 1b) and in its presence (Figs. 2a, 2b). Evidently, unlike the effect of N_2O which doubled the $[\dot{O}H]$, the introduction of the molecular oxygen did not affect the number of the primary lesions. Instead, the oxygen could suppress the restitution processes in the damaged biomolecules. In other words, in the case of the *exogenous* part of the *indirect* radiation effect, the molecular oxygen seems to interact directly with the target biomolecules, presumably by irreversible peroxidation of the primary lesions, thus competing with the restitution mechanisms.

Conclusions

In dilute buffer suspensions of T4 bacteriophage, where the *indirect*

radiation effect predominates, more than 90% of the radiodamage is caused by the *exogenous* water radicals. This phage inactivation is characterized by "multi-hit" dose-response curves which probably reflect the damage to the phage proteins.

In the presence of radical scavengers such as polyethylene glycol, the damage observed is practically the *endogenous* one. In this case, "single-hit" curves are obtained and probably reflect the damage to the viral DNA.

For the *exogenous* part of the damage, the $\dot{O}H$ is the reactive species among the primary water radicals whereas the contribution of the hydrated electrons to the biological inactivation is in fact negligible.

The superoxide radicals formed in the bulk suspension outside the phage do not seem to affect it. Even the combination of superoxide radicals and hydrogen peroxide does not enhance the phage inactivation, thus excluding the possibility that secondary $\dot{O}H$ radicals formed through the Haber-Weiss reaction contribute to the radiodamage.

The oxygen effect seems to result from the direct interaction of O_2 with the target biomolecules and a concurrent supression of the restitution processes.

References

1) G.E. Adams, Radiation chemical mechanisms in radiation biology. In Adv. in Radiation Chemistry (M. Burton and J.L. Magee, Ed.) Vol. III, Willey Interscience New York, pp. 125-208, 1972.

2) H. Dertinger and H. Jung, Molecular Radiation Biology, Springer-Verlag New York, pp. 70-113, 174-193, 1970.

3) S.A. Goscin and I. Fridovich, Superoxide dismutase and the oxygen effect, *Radiat. Res.* 56, 565-599 (1973).

4) G.P. Van der Schans and J. Blok, The influence of oxygen and sulfhydryl compounds on the production of breaks in bacteriophage DNA by γ-rays, *Int. J. Radiat. Biol.* 17, 25-38 (1969).

5) B.S. Srivastava, The yield of single-strand breaks in the DNA of bacteriophage λ irradiated extra and intracellularly with γ-rays in oxic and anoxic conditions, *Int. J. Radiat. Biol.* 26, 391-394 (1974).

6) B.S. Srivastava, Irradiation and DNA breaks, *Nature* 259, 425-426 (1976).

7) C.J. Dean, M.G. Ormerod, R.W. Serianni and P. Alexander, DNA strand breakage in cells irradiated with X-rays, *Nature* 222, 1042-1044 (1969).

8) C.D. Town, K.C. Smith and H.S. Kaplan, Influence of ultrafast repair processes on the yield of DNA single-strand breaks in E.coli K-12 X-irradiated in the presence or absence of oxygen, *Radiat. Res.* 52, 99-114 (1972).

9) I. Johansen and E. Boye, Radiation induced DNA strand breaks in E.coli measured within a fraction of a second, *Nature* 255, 740-742 (1975) and *ibid.* 259, 426 (1976).

10) J.J. Van Hemmen, W.J.A. Meuling, J. De Jong and L.H. Luthjens, Radioprotection of biologically-active DNA by cysteamin: A rapid mix study, *Int. J. Radiat. Biol.* 25, 455-464 (1974).

11) Z.M. Bacq and P. Alexander, Fundamentals of Radiobiology, pp. 45-69, 280-292, Academic Press New York, 1961.

12) V. Arena, Ionizing Radiation and Life, pp. 311-316, 444, Mosby St. Louis, 1971.

13) F. Haber and J. Weiss, Catalytic decomposition of hydrogen peroxide by iron salts, *Proc. Roy. Soc. London* A 147 , 332-351 (1934).

14) H.P. Misra and I. Fridovich, Superoxide dismutase and the oxygen enhancement ratio of radiation lethality, *Arch. Biochem. Biophys.* 176, 577-581 (1976).

15) I. Johansen, E. Boye and T. Brustad, Radiation induced strand break and time scale for repair of broken strands in superinfecting phage λ DNA in E.coli lysogenic for λ, in Fast Processes in Radiation Chemistry and Biology, L.H. Gray Fifth Conference, pp. 267-274, 1973.

16) M. Ikenaga, Comparative analysis of lethal lesions produced by ^{32}P decay and γ-rays in phage T1 and its host strains of E.coli with normal and reduced repair abilities, *Radiat. Res.* 34, 421-435, 1968.

17) T. Brustad, The effects of radical scavengers on the radiosensitivity of lysozyme in dilute aqueous solutions of varying pH, *Radiat. Res.* 27, 456-473 (1966).

18) M.S. Matheson, A. Mamou, J. Silverman and J. Rabani, Reaction of $\dot{O}H$ radical with polyethylene oxide in aqueous solutions, *J. Phys. Chem.* 77, 2420-2424 (1973).

19) J.D. Watson, The properties of X-ray inactivated bacteriophage, *J. Bacteriol.* 63, 473-485 (1952).

20) D. Freifelder, Mechanism of inactivation of coliphage T7 by X-rays, *Proc. Natl. Acad. Sci. USA* 54, 128-134 (1965).

21) G. Czapski and Y.A. Ilan, On the generation of the hydroxylation agents from superoxide radical - can the Haber-Weiss reaction be the source of $\dot{O}H$ radicals? *J. of Photochem. Photobiol.* (in press).

Table I Sensitivity to ionizing radiation of T4 bacteriophage γ-irradiated in 0.05 M phosphate: comparison of the 37% doses observed in the presence of various radical scavengers.

Experimental conditions	G (values of the water radicals) [radicals · (100 eV)$^{-1}$]				$D_{37\%}$ (Krad)
	H	$\dot{O}H$	e^-_{aq}	$\dot{O}^-_2$	
Argon saturated	0.7	2.8	2.8	-	5.8
Air saturated	-	2.8	-	3.5	3.0
N_2O saturated	0.7	5.6	-	-	2.5
O_2 saturated (0.01 M formate)	-	-	-	6.3	32.1
N_2O saturated (0.05 M polyethylene glycol)	-	-	-	-	31.3
N_2O + air saturated (1:1 mixture)	0.7	5.6	-	-	1.2

Footnotes to Table I

a) Irradiation conditions were: room temperature, dose rate of 96 Krad·g^{-1}·h^{-1}, pH 7. 4×10^5 PFU·ml^{-1}.

b) The values of $D_{37\%}$ were derived from the semi-logarithmic plots of survival. For "multi-hit" curves, the $D_{37\%}$ values were evaluated from the apparent linear portion of the curve observed beyond the initial "shoulder".

Legend to Figures

Fig. 1 Effect of oxygen and superoxide radicals on radiation lethality. Survival curves of γ-irradiated T4 bacteriophage in 0.05 M phosphate, pH 7, (4×10^5 PFU·ml^{-1}), room temperature.

a) argon saturated suspension; b) air saturated; c) oxygen saturated, 0.01 M formate: (Δ) no superoxide dismutase added; (▲) with 20 μg·ml^{-1} superoxide dismutase.

Fig. 2 Survival curves of T4 bacteriophage γ-irradiated in the presence of nitrous oxide. Other experimental conditions as in Fig. 1.

a) N_2O saturated suspension; b) saturated with both air and N_2O (1:1 mixture); c) N_2O saturated, 0.05 M polyethylene glycol.

Figure 1

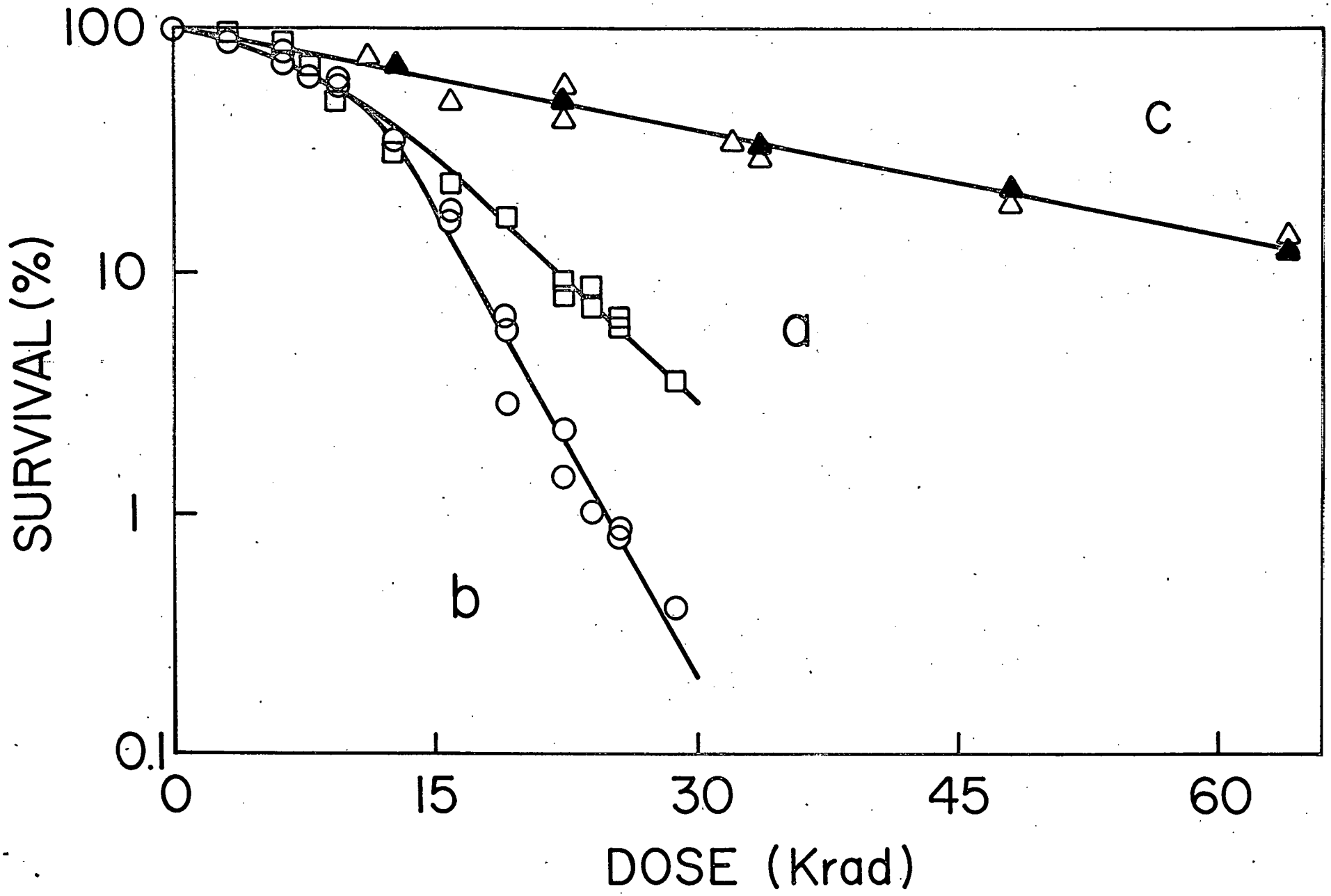

Figure 2