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MEASUREMENT OF THE EFFECTS OF X RAYS AND HELIUM IONS ON THE PROLIFERATIVE CAPACITY OF LYMPHOMA ASCITES TUMOR CELLS IN VIVO

Jose M. Tapia, John H. Lawrence, and Graeme P. Welch

November 22, 1967

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MEASUREMENT OF THE EFFECTS OF X RAYS AND HELIUM IONS ON THE PROLIFERATIVE CAPACITY OF LYMPHOMA ASCITES TUMOR CELLS *IN VIVO*[1]

Jose M. Feola, John H. Lawrence, and Graeme P. Welch

November 22, 1967

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ABSTRACT

Survival of lymphoma cells irradiated in special chambers under hypoxic and hyperoxic conditions has been used to calculate the oxygen enhancement ratio (OER) and the relative biological effectiveness (RBE) for X rays and He^4 ions of different LET. We have used 230-kV X rays (HVL = 1.6 mm Cu) and the He^4 ions accelerated by the 184-inch synchrocyclotron (LET's: 17 and 100 MeV-cm^2/gm) and by the 88-inch sector-focused cyclotron (LET's: 80 and 220 MeV-cm^2/gm) to irradiate the lymphoma cells. The oxygen enhancement ratio (OER) for X rays is 3.8 ± 0.9, and this value decreases constantly with increasing LET. For He^4 ions of 220 MeV-cm^2/gm the OER is 1.8 ± 0.5. RBE's calculated for both hypoxic and hyperoxic conditions are all close to 1.0, except for He^4 ions of high LET. The RBE for the He^4 ions from the 184-inch synchrocyclotron at peak is 1.8 ± 0.5. This figure is of particular interest in view of the extensive use of the Bragg peak in therapy. Comparing the OER for X rays with that for He^4 ions at peak (2.1 ± 0.7), a gain factor of 1.8 ± 1.0 is obtained. This gives an estimate of the decrease in the oxygen effect. Theoretical values of the gain factor for total destruction of the anoxic cells range from 2.5 to 3.5.

* * * * * *

KEY WORDS: OER, RBE, X rays, helium ions, Ascites, lymphoma

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INTRODUCTION

Although the greater biological effectiveness of heavy particles over electromagnetic radiation has been studied by J. H. Lawrence in normal tissues (1-3), as well as in neoplasms in animals (3-6), the need for a better understanding of the mechanisms of action of densely ionizing radiation, as well as the necessity of more precise measurement of the decrease of the oxygen effect with increasing linear energy transfer (LET), stimulated the initiation of a research program aiming at the clarification of these problems.

Studies of the biological effects of heavy ions on ascites tumor cells are particularly interesting because, unlike cultured cells, they are grown *in vivo*. In addition, parallel studies can sometimes be done by growing the same kind of cells *in vitro* as well as *in vivo*.

Quantitative studies of mammalian cellular radiobiology were limited to the precise techniques of culture *in vitro* until the methods first reported by Hewitt (7) and Hewitt and Wilson (8) allowed for measurement *in vivo* of cell viability by using cell dilution techniques.

Preliminary work done in this laboratory showed a greater RBE for the 910-MeV helium ions at the Bragg peak than the same particles at the plateau region[2] (9). Also, there was an indication that the oxygen effect was reduced to some extent, and the need for more quantitative studies was pointed out.

In the experiments reported here, improvements in the techniques used before have allowed us to make quantitative determinations of the oxygen enhancement ratio (OER) and the RBE of helium ions of different LET's. Our efforts in this direction are justified in view of the extensive use of helium ions in therapy (10-12).

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MATERIALS AND METHODS

The Tumor

A murine lymphoma (near diploid, s = 41) was used in the experiments reported here. This tumor line was obtained from Dr. D. B. Amos (13), and has been kept in ascites form by transplantation into A/Heston mice. This tumor "takes" in genetically closely related strains of mice such as A/Heston, LAF_1, A/JAX, and CAF_1. In these experiments we have used only LAF_1 female mice 12 to 16 weeks old and weighing 20 to 22 gm. The tumor-forming ability of the lymphoma cells (L#2), as defined by the TD_{50} (the number of cells that produces 50% of tumors within 8 weeks following intraperitoneal injection), has not changed in four years. It is: $TD_{50} = 10 \pm 4$ cells (standard deviation indicated).

The Irradiation Chamber

Figure 1 shows a cross section of a sample holder. Six were used and irradiated sequentially with the aid of a remotely controlled sample wheel. Each sample of ~71 mm^3 (~1.8×10^7 cells per chamber) was injected with a syringe through the 0.05-mm dialyzing paper cover into a 3.0-cm-diam by 0.1-mm-deep depression in the Lucite holder, and a 1/2-mil Mylar cover was placed over it. Neoprene "O" rings held the Mylar and dialyzing paper in place and sealed the cover to the holder.

For anoxic irradiations nitrogen gas was passed through a bubbler and flow gauge to a manifold to which six 4-foot lengths of 1/2-mm glass capillary were attached. The flow rates from the capillaries were checked for independence, then the capillaries were individually connected to the sample holders with small plastic tubing. At a flow rate

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of 50 ml/min, the system flushed out in about 1 min, and then the gas changed in each holder two to three times per minute. Twenty minutes were allowed for the oxygen in the samples to clear before the irradiations began.

For the hyperoxic irradiations pure oxygen was used and the same procedure was followed as described for hypoxic irradiations.

We have tried to keep the total flushing time for both hyperoxic and hypoxic irradiations at about 30 min, including the irradiation time. This was difficult in some cases when some technical problem arose after the flushing had been started.

Irradiations with the 88-Inch Sector-Focused Cyclotron

Irradiations with helium ions of 118 MeV initial energy were done at the 88-inch sector-focused cyclotron at the Lawrence Radiation Laboratory. At the irradiation position, 60 feet from the cyclotron, the beam was brought to a focus with a quadrupole magnet. Then, in order to cover the sample uniformly, a 0.05 mm Al scattering foil was put into the beam 29 feet upstream from the target position. The maximum nonuniformity of this beam was approximately 5%, as determined by a diametrical scan with a 1- by 1-mm diode.

Full beam energy of 118 MeV was used for the low-LET irradiations at 80 MeV-cm^2/gm. The low beam energy required for high LET irradiations was obtained by absorption in Lucite. Two remotely controlled wheels containing 1-1/2-inch-diam absorption discs were used first to obtain the Bragg curve, Fig. 3. Then the thickness which left a residual range of 0.09 gm/cm^2 was placed in the beam to give an LET of 220 MeV-cm^2/gm at the sample. From the slope of the Bragg curve

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cutoff and the sample thickness, it is estimated that the LET ranged from 200 to 260 MeV-cm^2/gm.

Doses were measured with a nitrogen-filled parallel-plate transmission ionization chamber with 6 μ aluminized Mylar electrodes. The electrodes were separated by 2 mm, and a 1-cm-diam circle was scratched on the aluminum surface of one of them to separate electrically the collecting electrode from its guard ring. The chamber's dimensions were measured with an optical comparator, and the dose corresponding to a microcoulomb of charge was calculated (14). A capacitor box and electrometer measured each irradiation's accumulated charge, from which the dose delivered was then determined.

The dose rate at the plateau region was 2800 rad/min with a standard deviation of 200 rad/min and a standard error of 110 rad/min. Extreme values of the dose rate were 2610 and 3100 rad/min. At the peak the average dose rate was 930 rad/min with a standard deviation of 800 rad/min and a standard error of 250 rad/min. Extreme values were 180 and 1990 rad/min.

Irradiations with X Rays and with the Helium Ions Accelerated by the 184-Inch Synchrocyclotron

X-ray irradiations were done with a 250-kV Philips machine, operating at 230 kV and 15 mA. Filtration of 1 mm Al and 0.25 mm Cu was used, and the HVL in Cu was determined to be 1.4 mm. At 30-cm TSD, the dose rate was 210 ± 10 R/min. Doses were measured with a 250-R Victoreen ionization chamber which had been recently calibrated by the National Bureau of Standards.

The 184-inch synchrocyclotron was used for irradiations with 910-MeV helium ions at the plateau region (LET = 17 MeV-cm^2/gm)

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and at the Bragg-peak region. The latter condition required 54 mm of copper absorber.

Raju has studied the energy distribution at the Bragg-peak position by using a 5-mm-thick silicon detector.[3] Figure 2 shows the energy distribution as well as the LET distribution. The average energy is 85 MeV, and the average LET is 100 MeV-cm^2/gm.

Dose rates have varied between 300 and 700 rad/min, with an average value of 540 rad/min and a standard deviation of 100 rad/min. Dose were measured as described above for the 88-in. cyclotron.

The Assay

Seven days prior to the irradiations, LAF_1 mice were injected with 10^6 L#2 cells. Thirty minutes before irradiation time these animals were sacrificed, and the tumor cells contained in the ascitic fluid were then placed in the thin chambers by injection with a syringe. The chambers were flushed for 20 min, and the irradiations performed under a constant flow of either nitrogen or oxygen. The cells from each chamber were recovered and placed in test tubes which contained 2 ml of tissue culture medium 199 (TC-199). Counting was done with the hemocytometer, and serial dilutions were prepared for injection. For each dose, as well as for controls, five groups of 10 animals each were intraperitoneally injected with 0.1 ml of fluid containing the different concentrations of cells. Factors of 5 were used between dilutions.

Injected mice were kept in plastic cages housing five mice each, and the animals were examined for tumors twice a week for a period of eight weeks.

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Surviving Fraction, Mean Lethal Dose, and RBE

The percentage of animals taking the tumor at each level of cell inoculation allowed calculation of the TD_{50} (the number of cells that causes tumors in 50% of the mice). Because of the low precision of the accumulation method of Reed and Muench (15) and the serious doubts raised regarding its appropriateness (16, 17), the Litchfield and Wilcoxon's method (18) was used to obtain the TD_{50}'s and their 95% confidence interval, with occasional control of results by means of probit analysis[4] (19).

The ratios of the TD_{50} for the control group to those for the different doses of radiation gave the surviving fractions (S. F.). Plots of surviving fractions as a function of dose gave the survival curves. Straight lines fit well most of the experimental curves. When shoulders are present, the extrapolation numbers range from 1.2 to 1.5.

The mean lethal dose, D_0, was used to estimate the RBE. The mean lethal dose is the dose in rads required to reduce to 0.37 the surviving fraction in the exponential region of survival curves.

The Gain Factor

The gain factor is defined as the ratio of the oxygen enhancement factor for X rays to that for helium ions. This factor indicates how much one "gains" by using the different helium ions when treating anoxic cells. For equal injury to well-oxygenated tissues, the effect on anoxic cells is increased as if the dose to these cells only had been increased in the ratio given by the gain factor (20).

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RESULTS

X-Ray Irradiations

Figure 4 shows the survival curves obtained by irradiation with X rays. The mean lethal dose is indicated in the figure, and all results have been summarized in Table I.

The mean lethal dose under hypoxic conditions was 380 ± 50 rads, and under hyperoxic conditions it was 100 ± 10 rads. The ratio of these two values gave an oxygen enhancement ratio of 3.8 ± 0.9 as indicated in the seventh column of Table I.

Points on both curves at the same dose level indicate values obtained in independent experiments. They show the degree of reproducibility that can be obtained when experimental conditions are well controlled. In the case of X rays, all laboratory procedures, irradiations, and injections were performed in the same building. When the irradiations were performed with the 184-inch synchrocyclotron or with the 88-inch sector-focused cyclotron, the timing of the experiments was more difficult, and greater variability was expected and actually observed.

Irradiations Performed with Helium Ions

184-inch synchrocyclotron: Figure 5 shows the survival curves obtained by irradiation of the lymphoma cells under hyperoxic and hypoxic conditions in the plateau region of the 910-MeV helium ions. For the hypoxic irradiation D_0 is 330 ± 50 rad; for the hyperoxic irradiation D_0 is 130 ± 30 rad. The ratio of these two quantities gave an OER of 2.5 ± 1.0.

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RBE's were estimated from the ratios of the mean lethal dose for X rays to that of the helium ions both under hypoxic and hyperoxic conditions. The values for 910-MeV helium ions were obtained the following way:

$$RBE\ (N_2) = 380 \pm 50\ /\ 330 \pm 50 = 1.2 \pm 0.3 \text{ (column 8, Table I)}$$

$$RBE\ (O_2) = 100 \pm 10\ /\ 130 \pm 30 = 0.8 \pm 0.3 \text{ (column 9, Table I).}$$

Finally, the gain factor was obtained as the ratio of the oxygen enhancement ratio for X rays to that for the helium ions. In the case we are exemplifying, the gain factor is

$$GF = 3.8 \pm 0.9\ /\ 2.5 \pm 1.0 = 1.5 \bullet 1.0 \text{ (last column, Table I).}$$

Survival curves for the Bragg peak of these particles are shown in Fig. 6, and the estimated values of D_0, OER, RBE, and GF are given in Table I. The energy and LET are the averages found by Raju[3] as shown in Fig. 2.

88-inch sector-focused cyclotron: Figure 7 shows survival curves obtained by irradiation of the lymphoma cells in the plateau region at an energy of 118 MeV, and Fig. 8 shows the survival curves obtained by irradiation at the Bragg peak. The shape of the Bragg curve for these particles can be seen in Fig. 3. Results have been summarized in Table I.

DISCUSSION

The end-point of these studies was the tumor-forming ability of the lymphoma cells. No matter by which mechanisms the tumor-forming ability was impaired, it showed up in the slope of the survival curves. When the irradiations were performed under hyperoxic conditions, the same dose of different kinds of radiation impaired the tumor-forming

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ability to the same proportion, within the limits of the experimental errors.

The presence of oxygen seems to make the impairment of the tumor-forming ability a function only of the dose given. On the other hand, survival curves obtained under hypoxic conditions showed a clear dependency on the LET of the helium ions used, in spite of the short span of the LET's explored in these experiments. The RBE's and gain factors calculated for the helium ions quantitatively showed the greater effectiveness of the Bragg peak as compared with the plateau and X rays, as well as the partial overcoming of the oxygen effect.

The assay we have used "certainly measures the net result of a number of radiation-initiated processes, all of which can lead ultimately to the failure of a cell to carry out unlimited proliferation" (21).

Qualitative differences between the action of alpha particles and X rays on lymphoma cells in vitro have been pointed out by Alexander (22). He also thought it was unlikely that DNA synthesis was involved in the events leading to cell death, since DNA continued to be made at the normal rate for many hours by the irradiated cultures.

Bacq and Alexander have indicated that "the fact that oxygen enhances the biological damage of radiations with low LET could arise from the fact that an isolated ionization initiates an autoxidation chain, thereby breaking these barriers which are made up in part of phospholipids in the same way as do several ionizations close together" (23). This would explain why the mean lethal dose under hyperoxic conditions is the same regardless of the type of radiation used. In all cases enough damage is produced, although by different mechanisms, to result in

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similar impairment of the proliferative capacity. The fact that densely ionizing radiation has a higher RBE than Co^{60} gamma rays has already been shown by Loughman et al. for these lymphoma cells using polyploidy induction as an end point (24), and by Feola et al. (25) using the same methods and end point as in this paper. This increasing RBE with increasing LET showed up when the irradiations took place under hypoxia. In the absence of oxygen, helium ions are more effective, not only in causing chromosome damage, but also in producing malfunction of the cell membrane, damage to the organelles, release of proteolytic enzymes, etc. All these effects would impair the ability of the cell to proliferate, and then would be measured in an assay for loss of proliferative capacity (21).

Small shoulders are present in some of the survival curves. The extrapolation number goes from 1.2 to 1.8 in those cases, in agreement with nearly shoulderless survival curves obtained by Bush and Bruce (26) and Silini and Maruyama (27) with lymphoma cells.

Comparison of the mean lethal doses obtained by us by irradiating in vitro with values reported in the literature for in vitro and in vivo irradiations gives good agreement for hypoxic conditions. Berry and Andrews (28) reported a value of D_0 = 365 rads for the P-388 lymphocytic leukemia, Belli and Andrews (29) obtained a D_0 of 415 R for the P-388 lymphocytic leukemia irradiated in vivo as an ascites 7-day-old tumor, and Silini and Maruyama (27) gave a D_0 of 437 R for the LSA ascites lymphoma irradiated in the C57BL dead mice.

Mendelsohn has pointed out the small spread observed for different tumors and techniques, and has given an average value of 358 R for anoxic cells (30).

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Regarding the hyperoxic irradiations, Mendelsohn gave an average value of D_0 = 131 R, which compares well with the value obtained by us with X rays (D_0 = 100 ± 10 rads). One must be careful, however, in comparing results, that hyperoxic conditions were really obtained in each experiment.

The value of D_0 = 160 rads reported by Berry and Andrews (28) was obtained by injection of hydrogen peroxide prior to irradiation. This value, as well as the one reported by Silini and Maruyama (27) of D_0 = 170 R for in situ irradiations, seems to indicate that the cells were not well-oxygenated. This seems to be clear by comparing with the value reported by Belli and Andrews (29) for the 1-day-old tumor, namely, D_0 = 110 ± 11 rads (SD), which agrees well with ours. When the irradiation is performed under vigorous bubbling of oxygen (95% O_2, 5% CO_2), as in the experiments done by Bush and Bruce (26) irradiating lymphoma cells in suspension and assaying by the spleen-colony method, the conditions are closer to those in our chambers. And so we report the mean lethal dose for Co^{60} γ-ray irradiations: D_0 = 114 ± 4 rads.

Considering the complexity of the factors involved, we think all these results are in essential agreement, and they point out the need of measuring the oxygen pressure in vivo as well as in vitro. The careful work of Deschner and Gray (31) has shown the difficulties of this task, as well as its potential in radiation research, especially for measurements in the ascitic fluid.

We have done some preliminary experiments to correlate pO_2 measurements with mean lethal dose. We have done these measure-

ments *in vivo* introducing a Beckman microelectrode housed in an 18-gauge needle into the peritoneal cavity instead of taking samples of the ascitic fluid (27). Calibration at four points at 37°C gave a straight line in most cases. A survival curve for a 7-day-old tumor is shown in Fig. 9. The $D_0 = 260 \pm 40$ rads indicated that some oxygen was present, since the mean lethal dose for anoxic cells is higher. However, the value of 21.0 ± 7.0 mm Hg obtained by measuring 10 animals with tumors of the same age seems slightly high, and we are in the process of repeating these experiments in tumors from 3 to 8 days old.

The oxygen-enhancement ratio is known to decrease with increasing LET (32-35), and consequently the gain factor increases. The range of gain factors obtained by us (1.5 to 2.1) is of the same order as values reported by other workers who used fast neutrons in their experiments (33-36).

The method used by us was not sensitive enough to show differences due to dose rate, and the same was true regarding survival under hyperoxia. Differences may exist, as has been shown with more precise techniques (37, 38). The RBE values reported here are in the range expected on the basis of previous work done in this laboratory with mammalian cells *in vitro* (37), and with techniques similar to ours (9, 39).

CONCLUSIONS

Somebody has said that all experimental work should be considered preliminary and reported as such. We think this is true, at least for this paper. We are especially interested in the Bragg peak of the

910-MeV beam, because of its intensive use in therapy, and because of the need to clarify the role of the high-LET components in the RBE and GF obtained. Experiments are under way to compare the effects of this beam on lymphoma cells with the effects on an aneuploid ascites tumor.

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FOOTNOTES

1. This work was done under the auspices of the U. S. Atomic Energy Commission, American Cancer Society, and Office of Naval Research.

2. Y. Schmidlin, J. H. Lawrence, K. Sillesen, G. Welch, and J. Lyman, Effect of heavy particles on the proliferative capacity of ascites tumor cells (lymphoma) grown in vivo. In Semiannual Report, Biology and Medicine, Donner Laboratory, University of California, UCRL-11833, 1964, pp. 80-94.

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TABLE I

Effects of helium ions and X rays on lymphoma cells irradiated *in vitro* and grown *in vivo*

Accelerator	Radiation	LET $\frac{\text{MeV-cm}^2}{\text{gm}}$	Number of animals[a]	D_0 (N_2)	D_0 (O_2)	$\frac{D_0(N_2)}{D_0(O_2)}$ [b,c]	RBE (N_2)[c]	RBE (O_2)[c]	Gain factor[c,d]
250-kV X rays	230-kV X rays	15-30	1500	380±50	100±10	3.8±0.9	1	1	
184-inch synchro-cyclotron	910-MeV He ions, plateau	17	500	330±50	130±30	2.5±1.0	1.2±0.3	0.8±0.3	1.5±1.0
184-inch synchro-cyclotron	85-MeV He ions, peak[d]	100[e]	1000	210±30	100±20	2.1±0.7	1.8±0.5	1.0±0.3	1.8±1.0
88-inch cyclotron	118-MeV He ions, plateau	80	1000	330±70	115±25	2.0±1.2	1.2±0.4	0.9±0.3	1.9±1.1
88-inch cyclotron	36-MeV He ions, near peak	220	1000	220±20	110±20	1.8±0.5	1.9±0.4	0.9±0.3	2.1±1.1

[a] Each experiment involves about 250 mice.

[b] Oxygen enhancement ratio.

[c] Errors have been propagated.

[d] The ratio of the oxygen enhancement factor for X rays to that for helium ions.

[e] Averages of energy and LET distributions are indicated.

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FIGURE LEGENDS

Fig. 1. Irradiation chamber. The lymphoma cells are irradiated under a 0.05-mm dialyzing paper cover. A 13-μ Mylar cover is placed on top.

Fig. 2. Distribution of helium-ion energy and LET at the Bragg peak of the 910-MeV helium-ion beam.

Fig. 3. Bragg curve of 118-MeV helium ions accelerated by the 88-inch sector-focused cyclotron.

Fig. 4. Lymphoma cells irradiated with 230-kV X rays under hypoxic and hyperoxic conditions. Standard deviations are shown. Points at the same dose level show degree of reproducibility obtained.

$$D_0(N_2) = 380 \pm 50 \text{ rads; } n = 1.0.$$

$$D_0(O_2) = 100 \pm 10 \text{ rads; } n = 1.8.$$

In all figures, open circles indicate hypoxic conditions, and dots show hyperoxic conditions.

Fig. 5. Survival of lymphoma cells irradiated with the 910-MeV He^4 ions accelerated by the 184-inch synchrocyclotron at Berkeley. $n(N_2) = 1.0$; $n(O_2) = 1.7$.

Fig. 6. Effects of He^4 ions accelerated by the 184-inch synchrocyclotron on the lymphoma cells when irradiated at the Bragg-peak region (average energy 85 MeV). $n(N_2) = n(O_2) = 1.0$.

Fig. 7. Survival of lymphoma cells irradiated with 118-MeV He^4 ions accelerated by the 88-inch sector-focused cyclotron at Berkeley. $n(N_2) = 1.2$; $n(O_2) = 1.1$.

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Fig. 8. Survival of lymphoma cells irradiated at the Bragg-peak region of the He^4 ions accelerated by the 88-inch sector-focused cyclotron. $n(N_2) = n(O_2) = 1.0$.

Fig. 9. Survival of lymphoma cells irradiated *in vivo* (7-day old tumor) as compared with the irradiations performed *in vitro*. n (*in vivo*) = 1.2.

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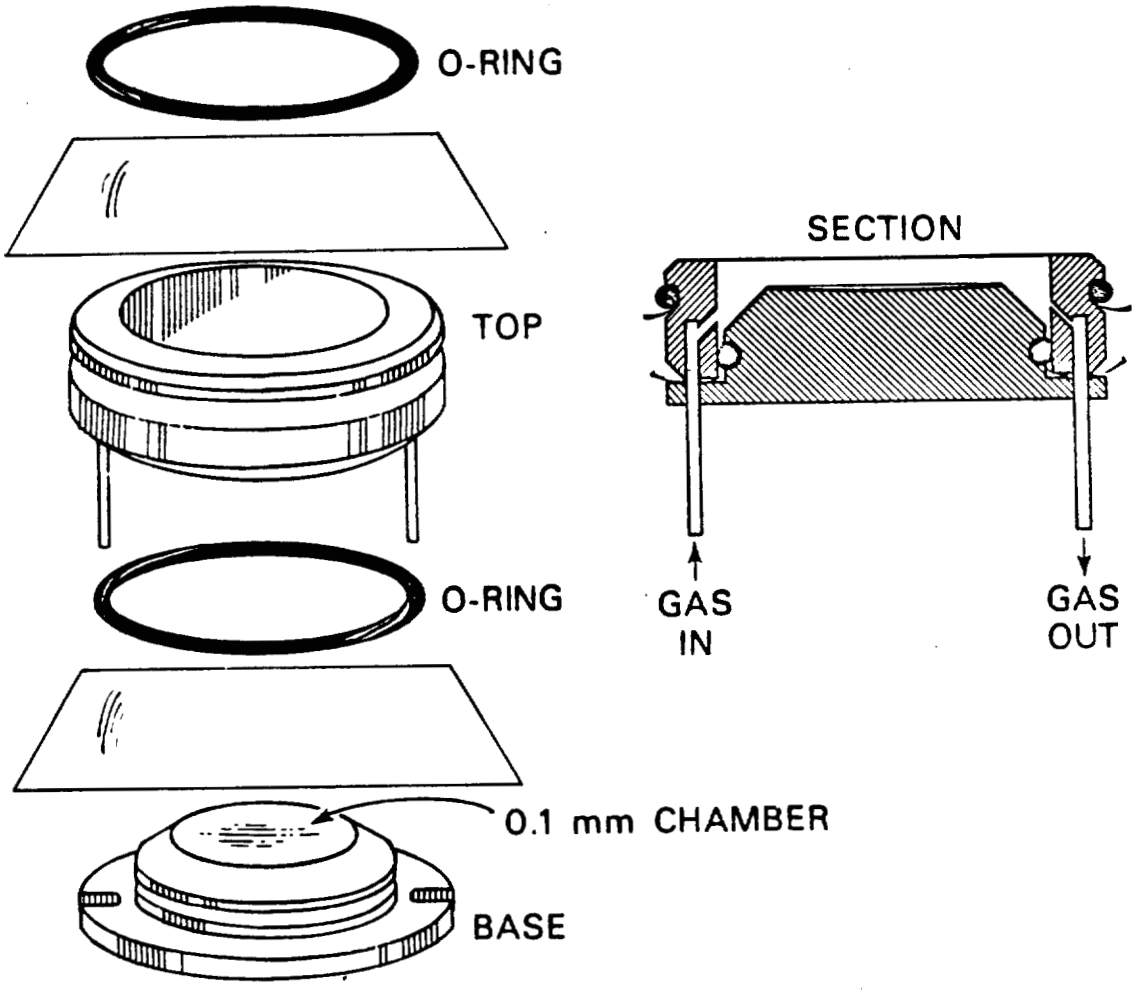

DBL 678-1772

Fig. 1

1175445

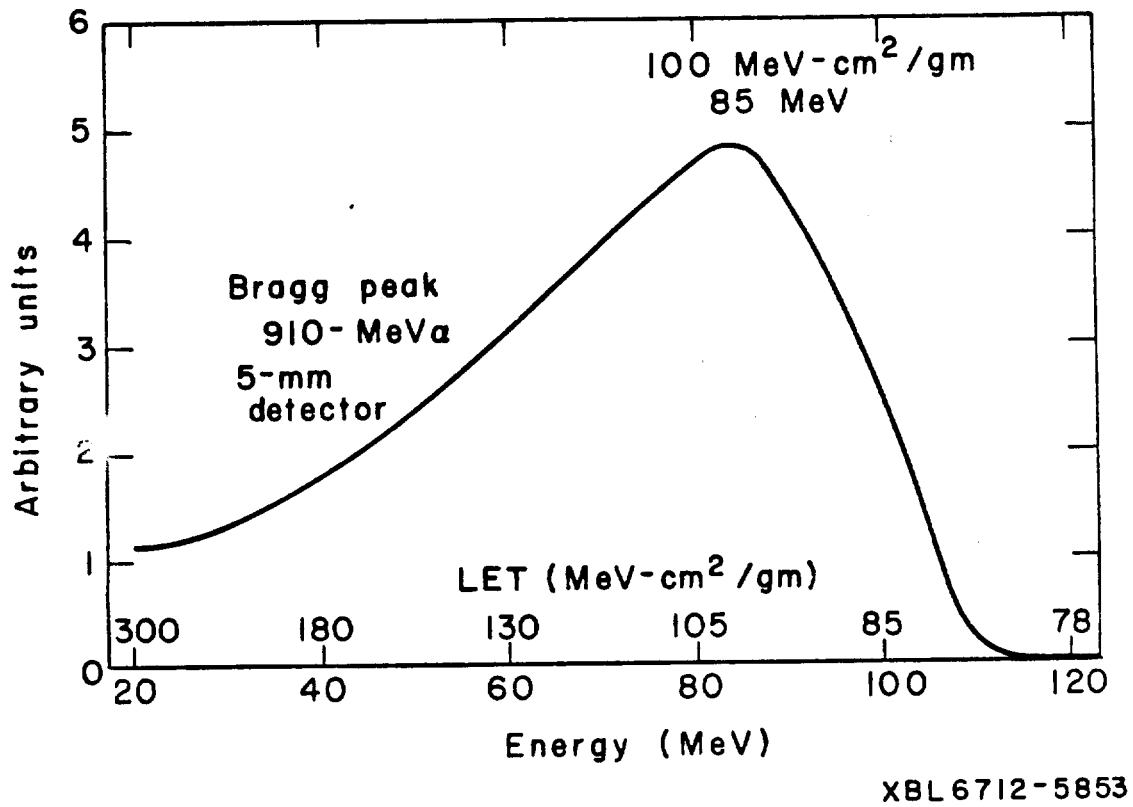

Fig. 2

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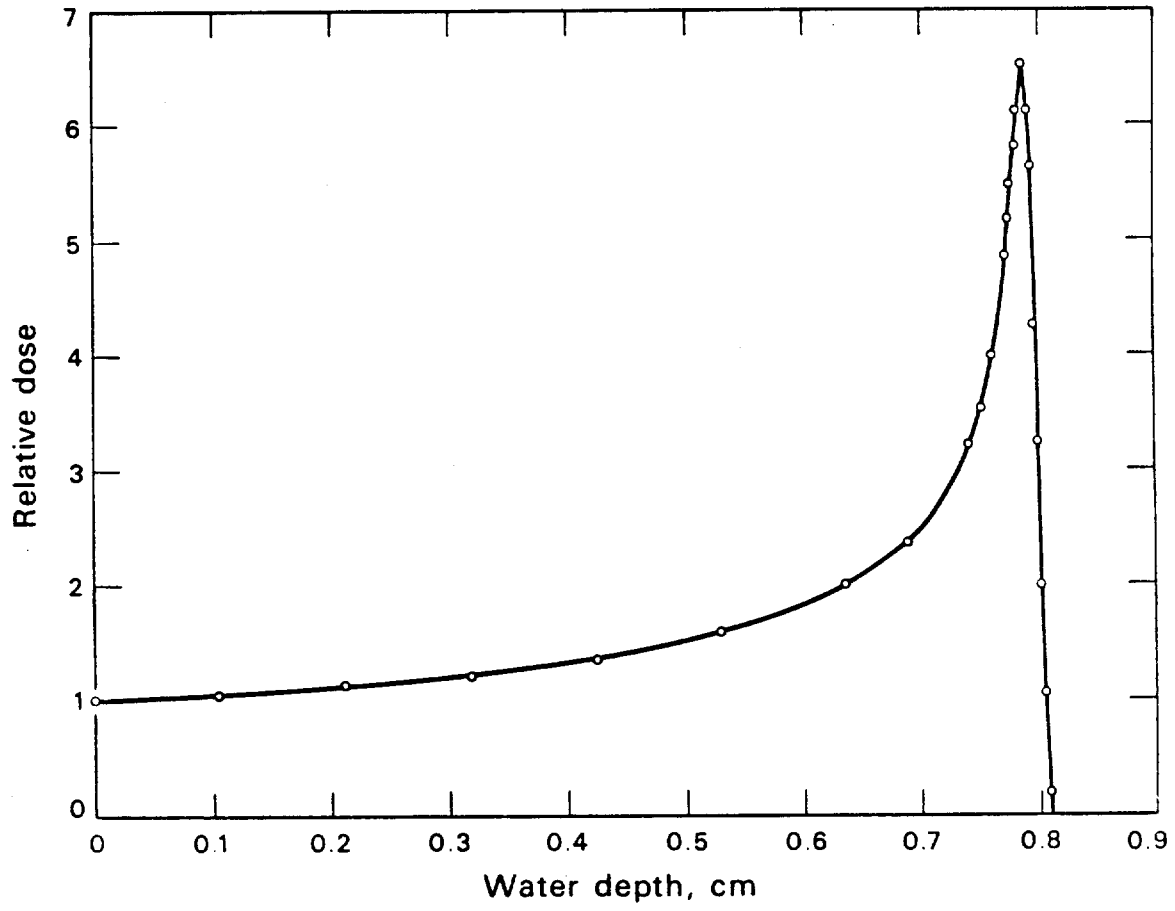

DBL 670-1838

Fig. 3

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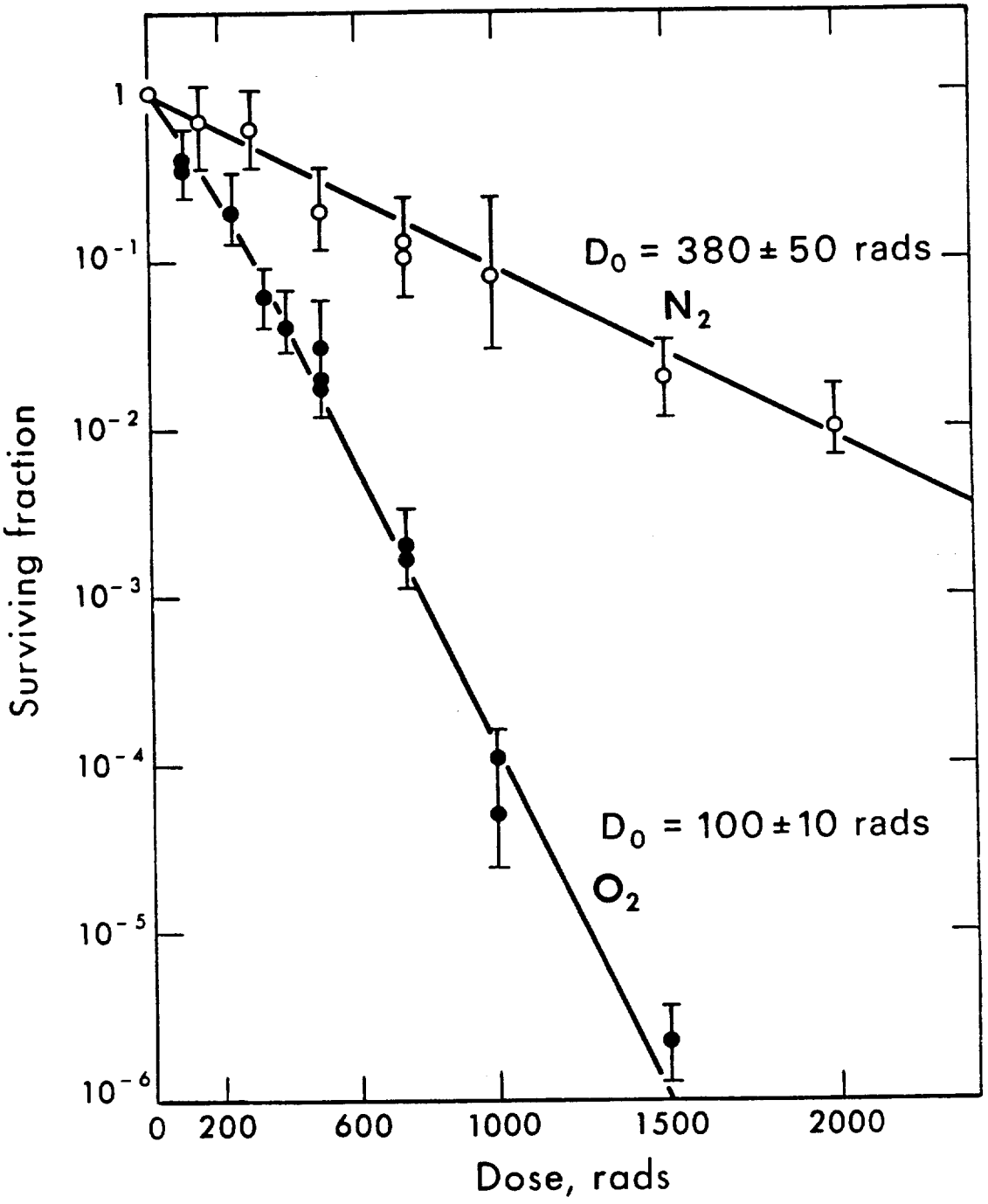

XBL672-777

Fig. 4

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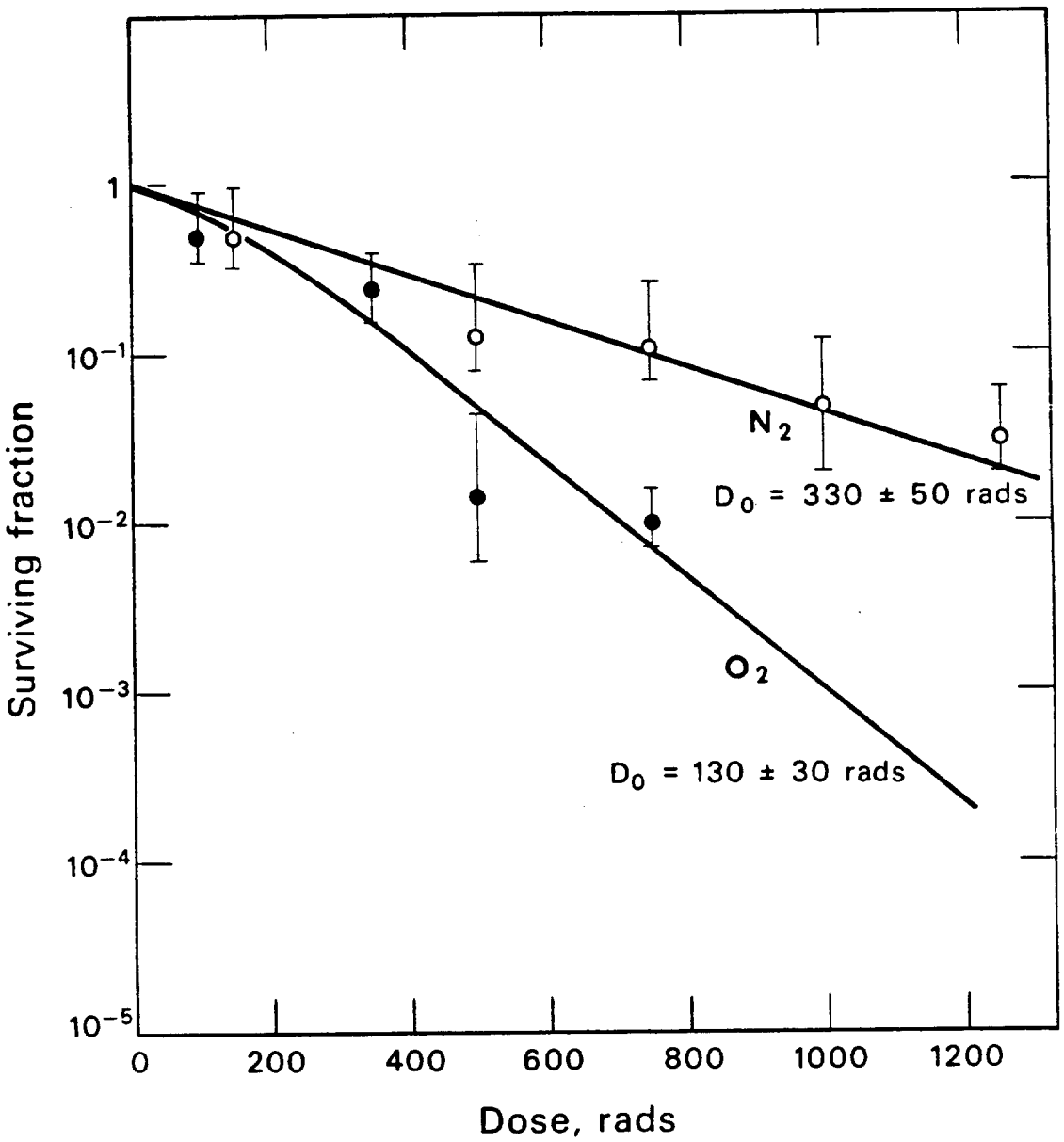

DBL 670-1830

Fig. 5

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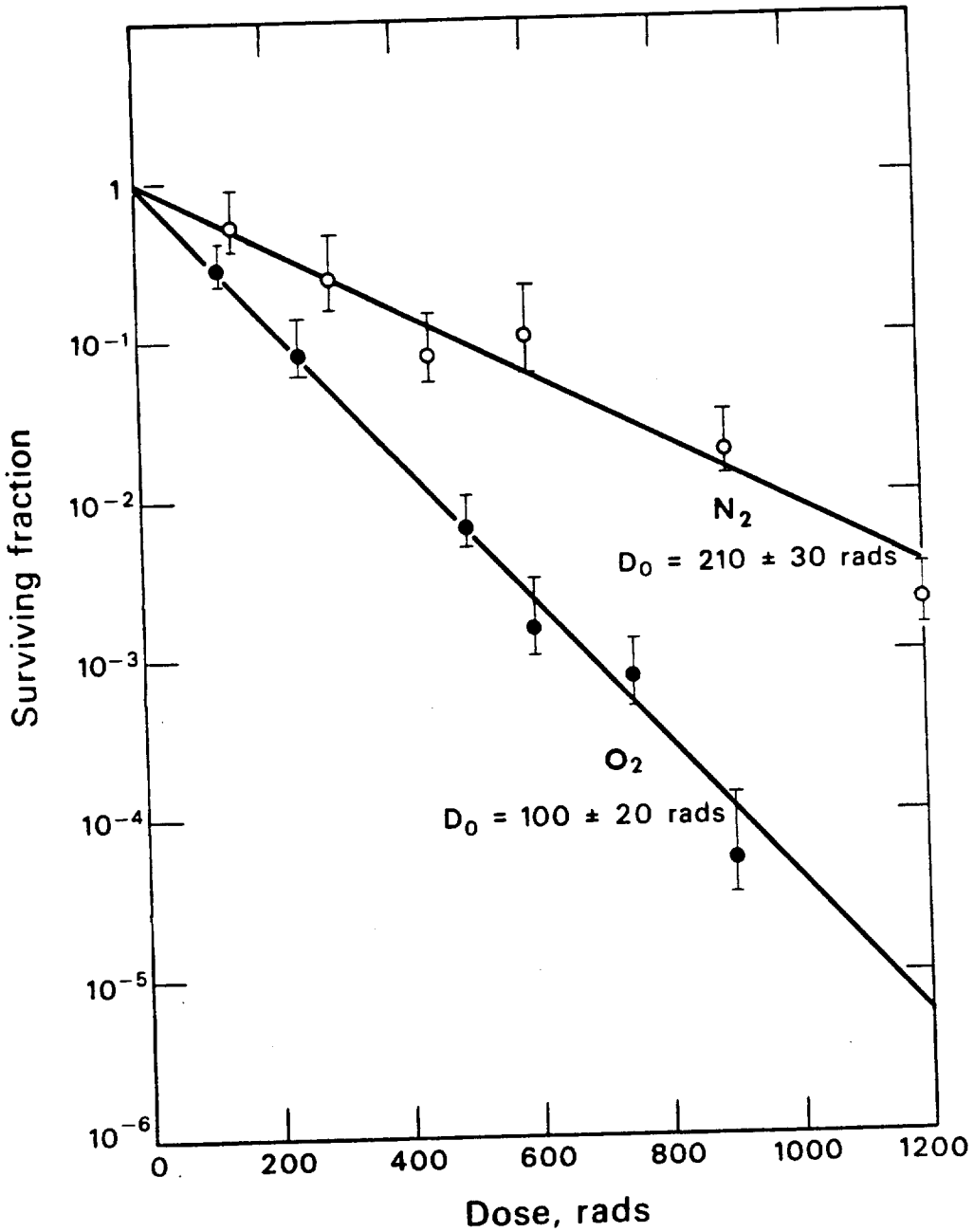

DBL 670-1831

Fig. 6

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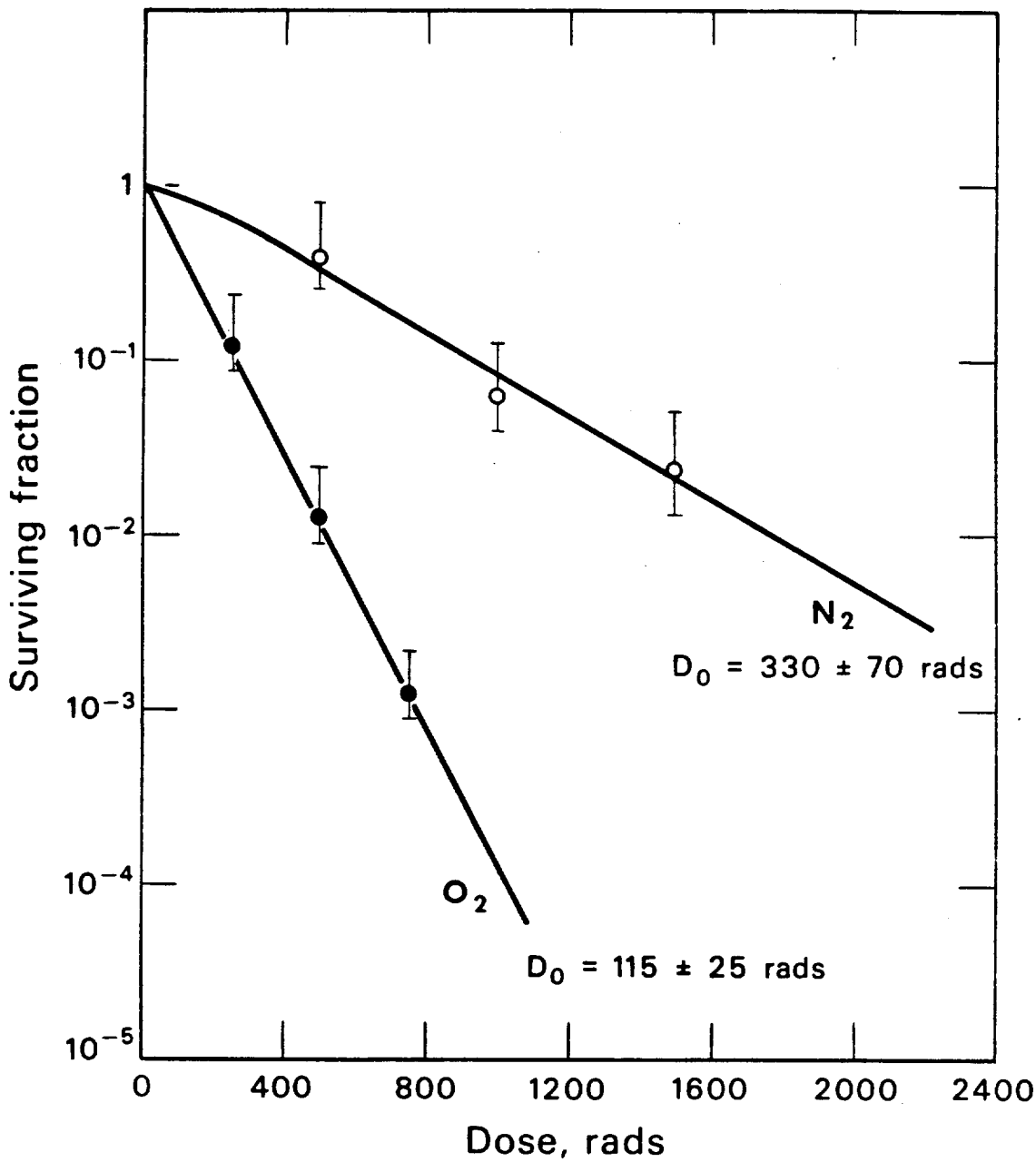

DBL 670-1829

Fig. 7

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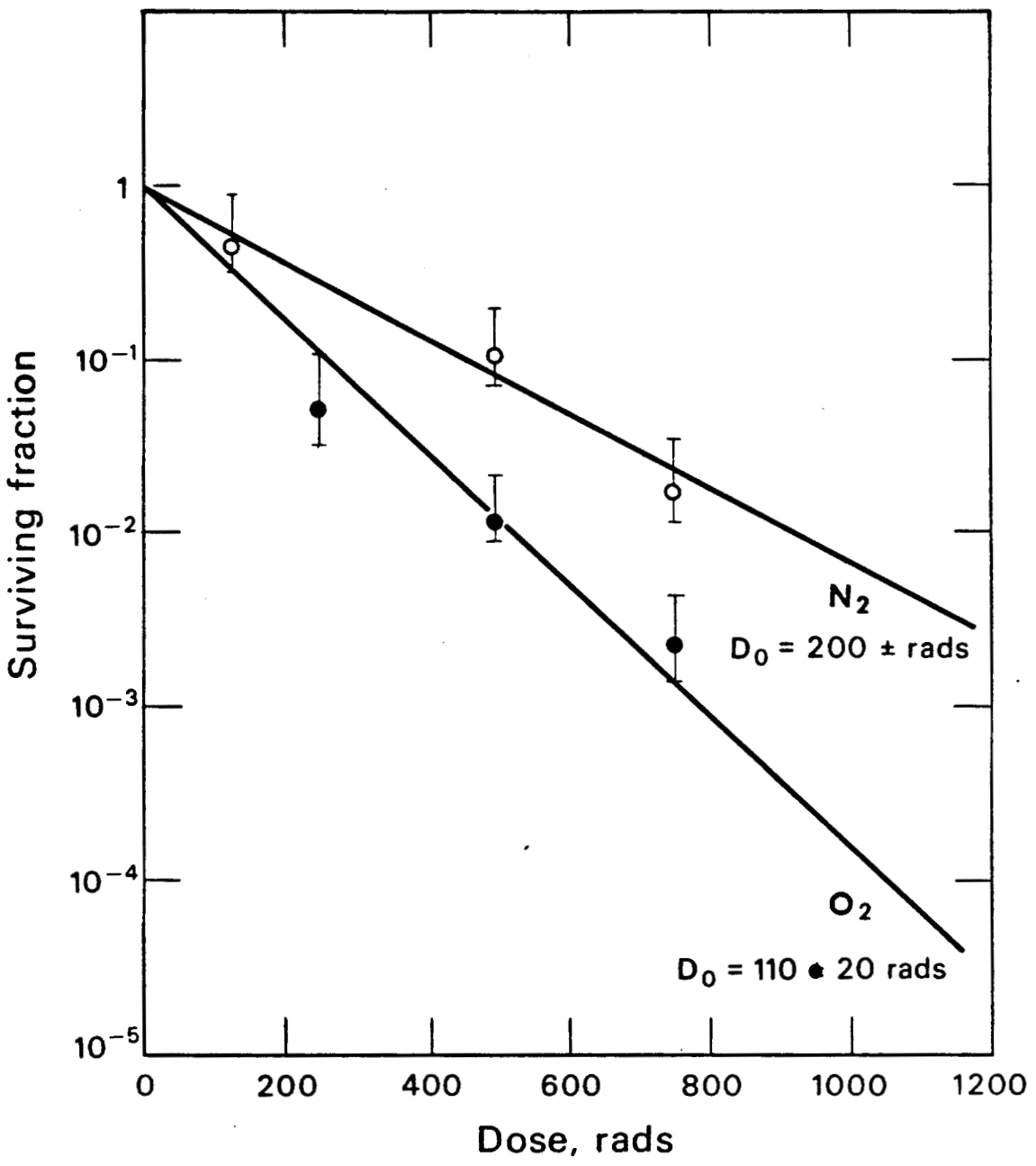
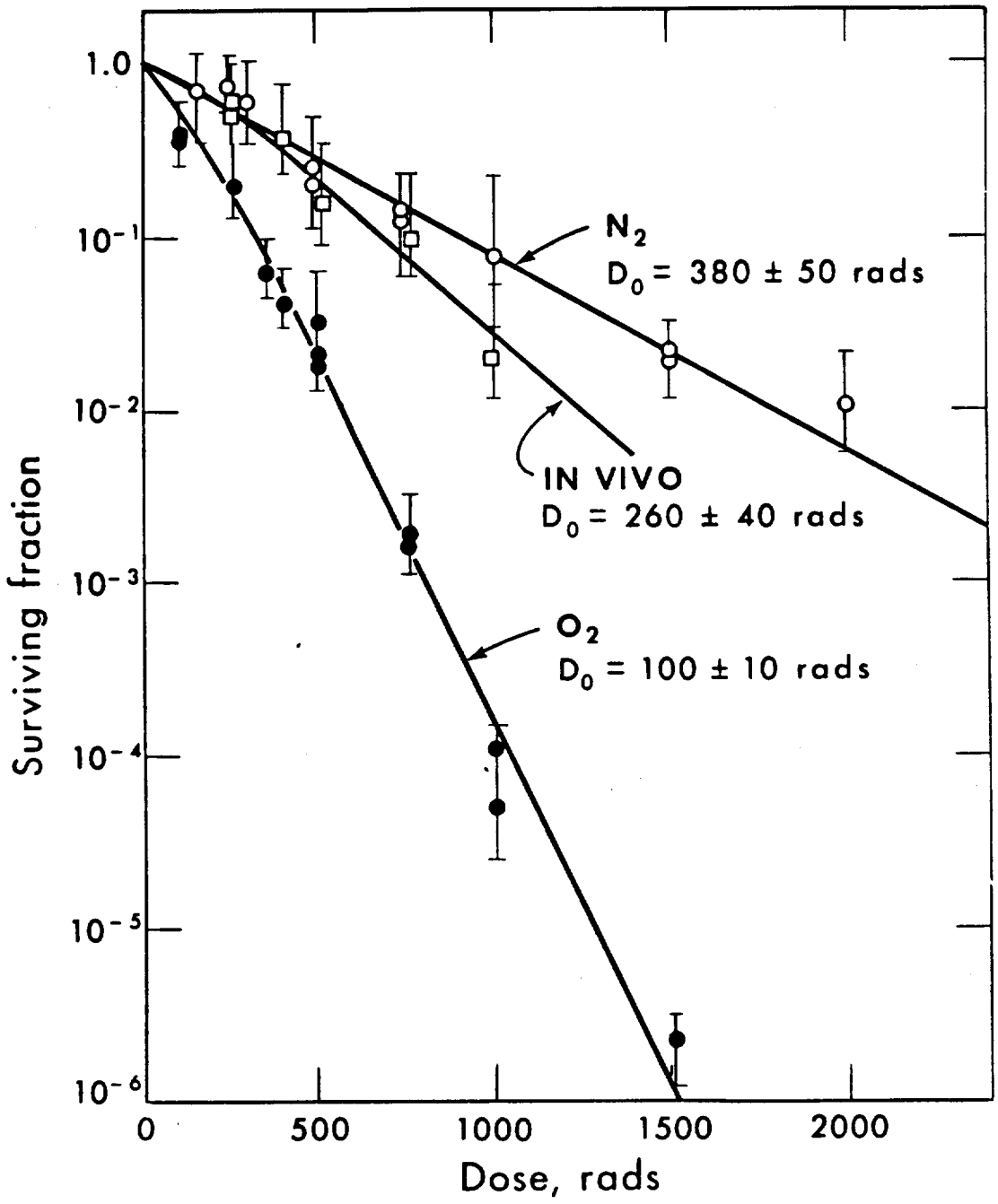


DBL 670-1832

Fig. 8

1175452



DBL 678-1769

Fig. 9

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