Title

LOW INCIDENCE OF POLYSPERMY IN DROSOPHILA MEIAN0GA3TER AND DROSOPHILA VIRILIS

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EFFECTS OF GLYCEROL AND OF ANOXIA ON THE RADIOSENSITIVITY OF HAPLOID YEASTS TO DENSELY IONIZING PARTICLES[1]

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GLYCEROL EFFECT AT HIGH LET

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INTRODUCTION

The exact roles played by oxygen and by water in affecting the sensitivity of biological systems to damage by ionizing radiation is of fundamental importance. As a thorough understanding of these two factors is needed to evaluate the relative importance of direct and indirect action, they have been extensively studied in many systems. (1,2)

In 1958, Wood and Rosenberg reported the use of hypertonic solutions to control the water content of haploid yeast cells.(3,4) They found that cells suspended in concentrated solutions (1 M to 6.9 M) of glycerol, glucose, ethanol, or methanol were less sensitive to inactivation by X rays than cells suspended in M/15 phosphate buffer. Furthermore, the degree of protection was found to be a function of the solute concentration, and in part or totally additive with the protection resulting from anoxia.

Burnett et al. had found much earlier that such compounds protect Escherichia coli B/r against X rays (5), and Markovich reported experiments with E. coli K-12 (λ) in which protection by glycerol was not additive with that due to anoxia (6). More recently Dewey has studied glycerol protection of the bacterium Serratia macescens and found the effect to be independent of and additive with protection from anoxia (7).

Sayeg et al., using cyclotron-accelerated helium and carbon ions and polonium-210 α particles, measured the radiosensitivity of haploid yeast over a broad range of linear energy transfer (^{8}).

They found that the sensitivity passed through a maximum as the LET was increased and then dropped abruptly with the most densely ionizing radiations. Sayeg et al. also calculated the inactivation cross section from their data and found that it tended toward a constant value at the highest values of LET. We studied as a function of LET the separate and combined effects on the radiosensitivity of haploid Saccharomyces cerevisiae of (a) treatment, during irradiation, with 6 M glycerol, and (b) anoxia. Different LET values were obtained by using the Berkeley heavy-ion linear accelerator (Hilac), which has been used successfully to accelerate a variety of densely ionizing heavy-ion beams at dose rates adequate for larger-scale studies of these various effects (9).

EXPERIMENTAL PROCEDURE

Biological Materials and Methods

A strain of haploid Saccharomyces cerevisiae designated S288C (10) was cultured for 2 weeks on potato dextrose agar (Difco) at room temperature. The day prior to an experiment the cells were harvested, washed three times by centrifugation in M/15 KH_2PO_4, and suspended in the same buffer at a final concentration of $8X10^6$ cells/ml. This suspension was kept on a wrist-action shaker at room temperature. This method results in a cell population which is suitably uniform in radiosensitivity. There is no clumping, and less than 0.1% of the cells are budding (11).

The range in tissue of the heaviest ions used in the experiments, neon ions, is less than 0.3 mm., and the ionization density increases very rapidly as the end of the range is approached. Accurate dosimetry and uniform exposure therefore require that the cells be exposed to the beam in a monolayer. This requirement and the further necessity for

being able to vary both the atmosphere and the solution to which the cells are exposed are conveniently satisfied by a method described both by Powers (12) and by Hutchinson (13), which has been slightly modified (14). Just prior to the irradiation an aliquot of the above suspension was diluted with either glycerol or phosphate buffer. Samples of these suspensions were pipetted onto the surface of a 13-mm-diameter membrane filter (Millipore, type H A) previously cemented at the edges to a disk of blotting paper of the same diameter. The suspending solution was rapidly absorbed into the blotting paper pad, leaving the cells in the required monolayer on the surface of the filter. The pad was then moistened with the same solution (Fig. 1). Because of the high porosity of the filter the cells remained in contact with the solution, but the thickness of the layer of solution covering the cells was negligible.

The chamber used for exposing samples to the heavy ions in controlled atmosphere accommodates ten samples on an aluminum disk which can be rotated from the outside. (15) This makes it possible to change the sample to be exposed without opening the chamber. Control samples were placed in the chamber with each load. The chamber was flushed with moist air or with moist N_2 which had been passed over hot copper turnings to remove oxygen. When N_2 was used the chamber was flushed for at least 10 minutes before irradiation. In a typical load, five samples were exposed to glycerol during irradiation and five to buffer, with appropriate controls included in the chamber. One such load was exposed in an air atmosphere and one in N_2 atmosphere with each beam. This made possible the determination of survival curves under these four sets of conditions with two or three different beams in a single run, using the same yeast suspension.

Immediately after irradiation of a load each sample was resuspended in 0.5 ml of M/15 KH_2PO_4 and spread on yeast-extract (Difco) dextrose agar in a petri plate. After incubation for 24 hours at room temperature, survival was scored by microscopic counts of single cells and microcolonies. Cells that were able to form microcolonies of ten or more cells were scored as viable. Comparison experiments showed that this criterion gave lower absolute sensitivities than, but the same relative values as, those obtained by counting visible colonies. The same methods were used to measure survival with 50-kv X rays.

Radiation Sources

For most of this work the Berkeley heavy-ion linear accelerator (Hilac) was used. The Hilac accelerates ions up through atomic number 18 to energies of 10.4± .2 Mev per nucleon. For these studies beams of helium, boron, carbon, and neon were used, with a pulse-repetition rate of either 15 or 20 per second. The pulse duration was 2 milliseconds. The accelerated ions, with the exception of the very heaviest, are stripped of all electrons upon passing through matter. In addition to the Hilac beams, protons from the Crocker Laboratory 60-inch cyclotron were also used. With this variety of radiations a LET range exceeding two orders of magnitude was obtained. Experiments were also carried out using unfiltered X rays from a beryllium-window tube (Machlett OEG-60) operated at 50 kv and 25 ma. The dose rate at the position of the cells was about 250 r/sec.

Doses for the heavy ions were measured with a very thin ionization chamber situated a few millimeters ahead of the sample. The current from this chamber was integrated through precision condensers by a battery-powered electrometer with negative feedback. A detailed discussion of the heavy-ion dosimetry has been given by Brustad et al. (15). Calculated LET distributions for these beams have been reported by Fluke et al. (16) and

by Brustad (17). For our analysis δ rays are defined as secondary electrons ejected with energies greater than the δ-ray threshold, which in accordance with previous work (17) is set equal to 100 ev. LET refers to the amount of energy lost per unit track length of an ionizing particle in energy transfers below the δ-ray threshold. The term "stopping power," $\frac{dE}{dx}$, on the other hand, means the total amount of energy lost per unit track length of an ionizing particle.

Calibration of the 50-kv X-ray tube is discussed by Mortimer (18).

RESULTS

Typical survival curves obtained by using 50-kv X rays and neon ions are shown in Fig. 2. The curves are normalized for 100% survival at zero dose. Viability of unirradiated controls included in the chamber with each load varied between 50% and 98% and was most often around 90%. Viability of unirradiated control samples in glycerol generally ran a few per cent lower than for those in buffer.

Survival data for all experiments were fitted to exponential relationships of the form

$$S = e^{-\alpha D} \qquad (1)$$

where S is the fraction of cells able to form a microcolony of ten or more cells after receiving a dose D expressed in rad. The parameter α is a measure of the sensitivity of the cells and is equal to the reciprocal of the 37% survival dose.

The oxygen enhancement effect observed when cells were irradiated with 50 kv X-rays in the presence of glycerol (Fig. 2A) was previously reported by Rosenberg (4), who used 200 kv X-rays. The apparent absence of an oxygen enhancement effect in the presence of glycerol when charged particle beams

were used for irradiation is surprising. The effect was absent even with low LET protons (see table I). This difference may reflect the greater localized dose rate along the track of the heavy charged particles. It seems possible that glycerol in the cell might impede the diffusion of oxygen enough that localized anoxia results in the vicinity of the densely ionized track.

Figure 3 shows the radiosensitivity of cells exposed in air to 101-Mev carbon ions, as a function of the concentration of glycerol. As seen from the graph, with increasing glycerol concentration the protecting effect approaches a saturation value. Unless otherwise specified, a glycerol concentration of 6 M was used throughout this investigation.

Figure 4 shows the relationship between radiosensitivity and time of exposure to 6 M glycerol before irradiation. In this experiment the cells were applied to the filter in approximately 0.02 ml of buffer. The pad supporting the filter was saturated with glycerol solution, which immediately wet the cells. After an interval which was varied from 2 seconds to 2 hours the cells were irradiated with 50-kv X rays. The dose rate was adjusted by varying the distance between the samples and the X-ray tube so that the time of exposure was reduced to a few seconds. Each of the six sensitivities plotted is based on a separate dose-effect curve with at least three points. The absolute magnitude of the sensitivities in this particular experiment differs from the others described in this paper. This is due to the use of a different method for assaying survival. It was expedient for this experiment to use the more conventional method of dilution plating, in which the criterion for viability is formation of a visible colony on yeast extract-dextrose agar.

As seen from Fig. 4, the full protective effect of the glycerol treatment was not achieved until the cells had been exposed to the solution for

10 minutes. Osmotic shrinking of these cells, however, is virtually completed within 2 minutes. This was measured by centrifuging a cell suspension in a hematocrit tube before and after addition of glycerol. The cells initially shrank to about 65% of their original volume and then gradually swelled back to that volume during the following 65 hours. No cell multiplication occurred during this time.

Table I summarizes the present experimental values of α, determined under various experimental conditions and for different radiations. When a value was measured more than once the results were averaged for brevity in reporting. The standard deviations from these means are tabulated as error intervals. Where no error interval is given the point was measured only once.

An alternative relationship which is useful in analyzing survival data is obtained by expressing the dose as f, the number of particles per square centimeter incident on the sample. The survival curve is then of the form

$$S = e^{-\sigma f}, \qquad (2)$$

where σ is the effective cross section for inactivation. From the value of α in rad^{-1}, the quantity σ, in cm^2 per particle, may be calculated from (19)

$$\sigma = 1.602 \times 10^{-8} \left(\frac{dE}{dx}\right)\alpha, \qquad (3)$$

where $\frac{dE}{dx}$ is given in units of Mev g^{-1} cm^2.

In Fig. 5 the sensitivity α is plotted against the stopping power, $\frac{dE}{dx}$, for each particle on log-log scales, and Fig. 6 is a similar plot of the cross section σ, with the line of constant RBE shown for comparison.

Both the protection which results from anoxia and that due to the glycerol treatment are effective over the entire range of LET studied, even where the inactivation cross section has apparently reached a maximum. It does not appear (Fig. 6) that the magnitude of either of these effects would decrease at even higher LET's. The two effects are qualitatively and quantitatively differ-

ent, however. The oxygen effect is clearly a function of LET, being appreciably smaller with the more densely ionizing particles. Further, the magnitude of the oxygen effect at high values of LET is sufficiently small that it may possibly be entirely accounted for by the delta ray component of the heavy ion tracks (17). The glycerol effect, on the other hand, appears to be independent of LET and therefore not as easily explained. Because of apparent lack of an oxygen effect with glycerol, discussed above, it is probably safer to compare the data obtained anaerobically, with and without glycerol. Under these conditions we find that 6 M glycerol reduces the inactivation cross section by about 40% throughout the entire range of LET studied.

DISCUSSION

Howard-Flanders recently suggested a mathematical method to describe relationships between observed radiosensitivities and the ionization density (20). This method is based on the assumption that the lethal damage results from a process involving two or more steps. The first step is a direct interaction between the ionizing radiation and some critical molecules in the cell. The interaction leaves this molecule in a reactive excited state. There is a chance that this excited molecule will return to its normal state, in which case no damage results. Oxygen, if present, competes with this reverse reaction, resulting in an irreversible lethal damage as the second step of the process. Alternatively, the secondary irreversible step can be the combined result of several additional ionizations in the region immediately surrounding the injured molecule. This could occur independently of the presence of oxygen. In other words, the lethal damage under anoxic conditions is assumed to be the result of n or more

ionizations within the track segment of length t, whereas the oxygen-dependent component of the injury is assumed to be produced by single ionizations, according to a first-order approximation, in which all ionizations in the track segment t are equally effective up to n-1.

For a detailed analysis according to this theory, it is necessary to know

(a) the total LET-energy spectral distributions in the sample material of all the various radiations used,

(b) the probability per unit energy absorbed of having a certain number of ionizations in a track segment of length t, when the mean number in t is known.

Such calculations have been performed and their usefulness in describing the LET dependence of radiobiological effects discussed (17).

The curves in Fig. 7 have been calculated according to this method. It will suffice to note that the various curve shapes shown (and their displacement along the abscissa) are determined by combining the appropriate probability function (b) with the corresponding distributions in (a). The displacement of these curves along the ordinate axis is given by a factor--the sensitivity parameter k --characteristic for the system studied.

Table II shows the various parameters used in this track-segment analysis.

The radiosensitivities of cells exposed in 6 M glycerol, both in the presence of oxygen and under anoxia, depend in the same way on the LET of the radiations, without any oxygen effect for the particle radiations used. The injury can be described as the result of 10 or more ionizations formed within a track segment of length about 70 Å, with a sensitivity parameter of 14×10^{-4} rad^{-1}.

The LET dependence of the radiosensitivity of cells exposed in phosphate buffer suspension under anoxia also showed the same general trend. In Fig. 7 these experimental points are fitted by assuming the injury to be the result of 10 or more ionizations within 70 Å, with a sensitivity factor of 26×10^{-4}, or 1.9 times as high as after glycerol treatment.

In other words, it has been demonstrated that the glycerol treatment described here results in a radioprotection, which is essentially independent of LET. Here, perhaps, is an additional test that can be applied to any proposed model for radiobiological action. It is of particular interest to ask whether this effect might be more reasonably explained on the basis of modification of direct action of the radiation on some sensitive site in the cell, or on the basis of indirect action. It is worth noting that in our analysis we have considered the effects of the entire LET-energy distribution of the various radiations, and the result does not depend on any δ-ray correction.

Our analysis describes fairly well the LET dependence of the radiosensitivities of cells exposed in glycerol solution, irrespective of gas atmosphere during irradiation. The agreement for cells exposed in phosphate buffer under anoxia is not a good at the highest LET.

Calculations based on the proposed model to fit the observed sensitivities in phosphate buffer in air atmosphere, however, led to a curve which did not even approach the general trend of the experimental points. This discrepancy demonstrates clearly the insufficiency of the model.

A possible though not very attractive solution is to introduce another parameter, ω, which allows the oxygen-dependent mechanisms to operate with only a fraction of the efficiency of the oxygen-independent mechanisms(17). The curve marked "air-buffer" is thus calculated by assuming that the oxy-

gen-dependent part of the injury is the result of one to nine ionizations within 70 Å, with $\omega = 0.2$.

An underlying assumption of the Howard-Flanders theory is that increasing LET leads to saturation of the effects. Thus, if this theory is true, one would conclude that 10 or more ions in a track length of 70 Å are effective over a cross section of 8×10^{-9} cm^2 when the cells are irradiated anoxically in buffer, but that in the presence of glycerol the same number of ions in that same track length is effective only over a cross section of about 5×10^{-9} cm^2. There are some ions, then, that pass through the cross-sectional area which are effective in the presence of buffer but ineffective when glycerol is present. However, any mechanism that deals with a direct hit as the primary effect should result in the same cross section for very high LET, regardless of the presence of a modifier.

The only parameter in this analysis which reflects the glycerol effect is the "sensitivity factor" k. The physical significance of this parameter is not explicitly defined in Howard-Flanders' model (20, 17), as indeed his model does not predict the absolute sensitivity, but rather only the relative sensitivity as influenced by LET and oxygen concentration. It is therefore of interest to examine our results for a possible physical interpretation of the magnitude of the sensitivity of this particular system.

Before considering such interpretation it may be instructive to review briefly the accumulated evidence against the interpretation that glycerol protects by removing the bulk water from the cell.

A. We found that the onset of protection after immersion in glycerol occurred much later than the removal of cellular water, as reflected in the change of packed cell volume. It appears that, at least in part, the protection is related to the actual presence of glycerol in the cell.

B. Rosenberg has reported X-ray studies on yeast using, not only glycerol but also methanol, ethanol, sucrose, and potassium chloride (4). Markovich, working with the lysogenic bacterial system E. coli K-12 (λ), measured the X-ray protecting ability of a number of water-soluable organic compounds, including, in addition to those used by Rosenberg, mannitol and acetone (6). All these compounds, with the exception of KCl and acetone, were found to have a similar protective effect. Those tested with yeast gave the same degree of protection on a molar basis. Although all these agents can be considered to dehydrate the cell, the mode of action differs. Those which enter the cell slowly (compared with the rate at which water leaves) shrink the cell initially. Others (methanol and ethanol) do not cause appreciable shrinking. It is of possible interest to observe that of these compounds, the ones which protect are also those which contain OH groups.

C. Wood recently reported that desiccated yeasts have about the same sensitivity to X-rays as yeast suspended in water (21).

D. Webb and Powers (22) found a glycerol protection effect for spores of Bacillus megatherium, exposed to X rays. Desiccation of these spores, however, makes them more sensitive, just the opposite effect from glycerol.

These observations, however, do support the idea that while not all the cellular water is radiobiologically active, a small fraction of it is involved. The water most generally implicated is the bound water, or water of crystallization associated with critical proteins and nucleic acids. It is this water which would not be removed by osmosis or by gentle desiccation and which might play a paramount role in energy-transfer processes in the immediate vicinity of these molecules. This would also be consistent with the negative results obtained by Rosenberg with KCl and the positive results with alcohols and sugars. This interpretation would be consistent with pro-

tection at very high LET, since it has been shown that intermolecular energy-transfer processes contribute significantly to inactivation of dried proteins by heavy ions (14).

An alternative explanation, then, would be that at least part of the significant primary interaction is with molecules external to the critical molecules in question (23). We may consider the curves in Fig. 6 to be of the general form

$$\sigma\ (\mathrm{LET}) = F(\mathrm{LET})\ \sigma_o$$

where σ(LET) is the observed inactivation cross section at any particular LET and F(LET) is an efficiency function analogous to the curves in Fig. 7. The limiting cross section, σ_o , characterizes the sensitivity of the system and is thereby analogous to the "sensitivity factor" k. Comparing the curves obtained in the absence of oxygen, with and without glycerol, then, it is evident that the function F(LET) is not affected by glycerol. The two curves can be made to coincide by simply multiplying σ_o of the glycerol curve by 1.75. The fact itself that σ_o can be modified by glycerol suggests that the physical significance of this quantity cannot be that it represents the physical dimensions of some critical target in the cell. While the dimensions of critical molecular groupings are of obvious importance, it appears that a substantial part of σ_o is related to the distance over which intermolecular energy transfer processes are effective. It would then appear that glycerol and related substances exert their protective effect by inhibiting some of these processes.

Independent evidence supporting this interpretation has been presented by Webb (24), who has reported that "the dependence of X-ray sensitivity on glycerol concentration is fitted well by the Langmuir adsorption isotherm

equation." On this basis he suggests that glycerol acts by competing with water for adsorption sites at the surface of macromolecules.

This interpretation suggests a need for experiments designed to elucidate the role of water in energy transfer processes in macromolecular systems and the role of these in cellular radiobiology.

SUMMARY

The radiosensitivity of haploid yeast (Saccharomyces cerevisiae) has been measured over the range of LET from 20 Mev g^{-1} cm^2 to 5500 Mev g^{-1} cm^2 under four sets of conditions:

(a) air atmosphere in buffer solution,

(b) N_2 atmosphere in buffer solution,

(c) air atmosphere in 6 M glycerol solution,

(d) N_2 atmosphere in 6 M glycerol solution.

Treatment with 6 M glycerol was found to protect cells irradiated in anoxia by an additional factor of about 1.9 throughout the range of LET studied. Experiments designed to study the kinetics of this effect were performed. The maximum protective effect was not seen until the cells had been exposed to glycerol for about 10 minutes, whereas the water was apparently removed much faster. The results are discussed in relation to current models of radiobiological action. A comparison is made with results reported by other experimenters.

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Table I. Sensitivities of haploid yeast to heavy ions under various conditions.

Radiation	Stopping power, $\frac{dE}{dx}$ ($Mev\ cm^2/g$)	Reciprocal of 37% survival dose ($10^{-4}\ rad^{-1}$)			
		Air-buffer	N_2-buffer	Air-glycerol	N_2-glycerol
50-kv X rays	20	1.54 ± .01	0.79	0.63	0.46
11-Mev protons	45	1.47	0.78	0.39	0.40
39-Mev He ions	182	1.37 ± .12	0.73 ± .17	0.42 ± .11	0.47 ± .10
30-Mev He ions	610	1.86 ± .20	1.6 ± .5	1.0 ± .4	0.87 ± .34
98-Mev B ions	1250	2.78	2.35	1.34	1.32
84-Mev B ions	1485	2.74			
101-Mev C ions	1850	2.7 ± .4	2.1 ± .3	1.2 ± .1	1.17 ± .16
142-Mev Ne ions	5500	1.20 ± .15	0.9	0.57	0.56

Table II. Parameters used in the track-segment analysis in this study.

Medium in which cells were exposed	Gas present during the exposure	Number of ionizations per trach segment of length t	Track length t (A/ρ)	Sensitivity parameter $K(rad^{-1})$	ω
6 M glycerol	N_2	10 or more	69	14×10^{-4}	
6 M glycerol	Air	10 or more	69	14×10^{-4}	
PO_4-buffer	N_2	10 or more	69	26×10^{-4}	
PO_4-buffer	Air	1 to 9	69	26×10^{-4}	0.20

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FIGURE LEGENDS

Fig. 1. Schematic diagram illustrating procedure used to control atmosphere and solution to which yeast were exposed during irradiation with heavy ions.

Fig. 2. The effects of anoxia and 6 M glycerol on the survival of haploid yeast as a function of absorbed dose for two radiations: (A) 50-kv X rays, and (B) 142-Mev neon nuclei. The curves are normalized for 100 % survival at zero dose. Error intervals are standard deviations.

Fig. 3. Radiosensitivity, as a function of time in 6 M glycerol before irradiation, of haploid Saccharomyces cerevisiae exposed in air to 101-Mev carbon nuclei.

Fig. 4. Radiosensitivity, as a function of time in 6 M glycerol before irradiation, of haploid Saccharomyces cerevisiae exposed in air to 50-kv X rays.

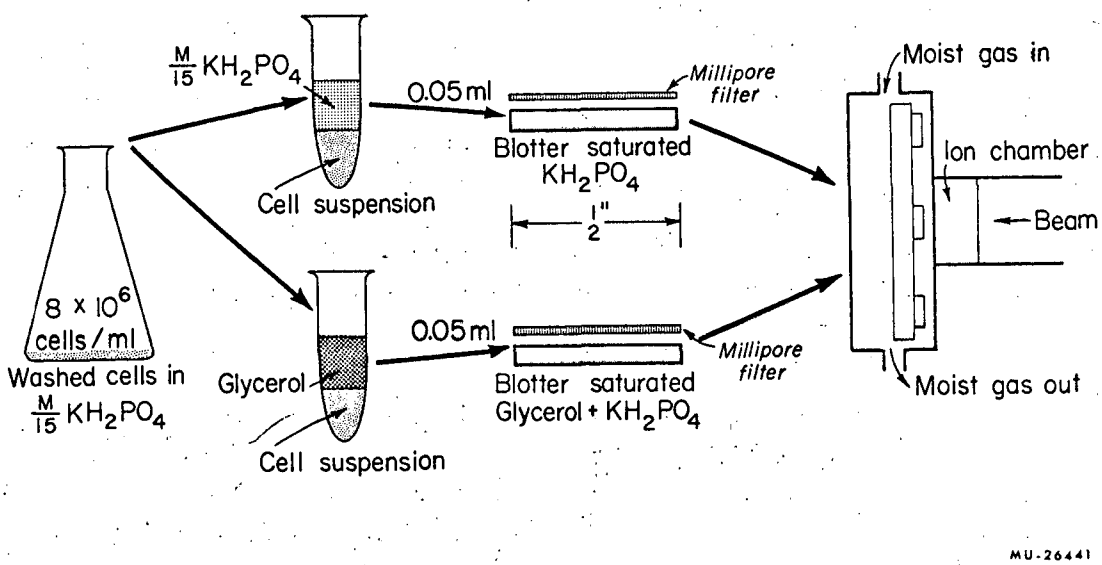
Fig. 5. The radiosensitivity, as a function of the stopping power, $\frac{dE}{dx}$, for the various radiations used, of haploid Saccharomyces cerevisiae under various conditions.

Fig. 6. The cross section for inhibition of microcolony formation of haploid Saccharomyces cerevisiae exposed to heavy ions under various conditions. The continuous lines are merely drawn to connect the experimental points.

Fig. 7. Comparison of theoretical curves calculated by track-segment method from the data in Table I. See discussion for details.

FOOTNOTES

1. This study is based on work performed under contracts with the U.S. Atomic Energy Commission.
2. Present address: Norsk Hydro's Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway.


M/15 KH2PO4
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Moist gas out
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Figure 1

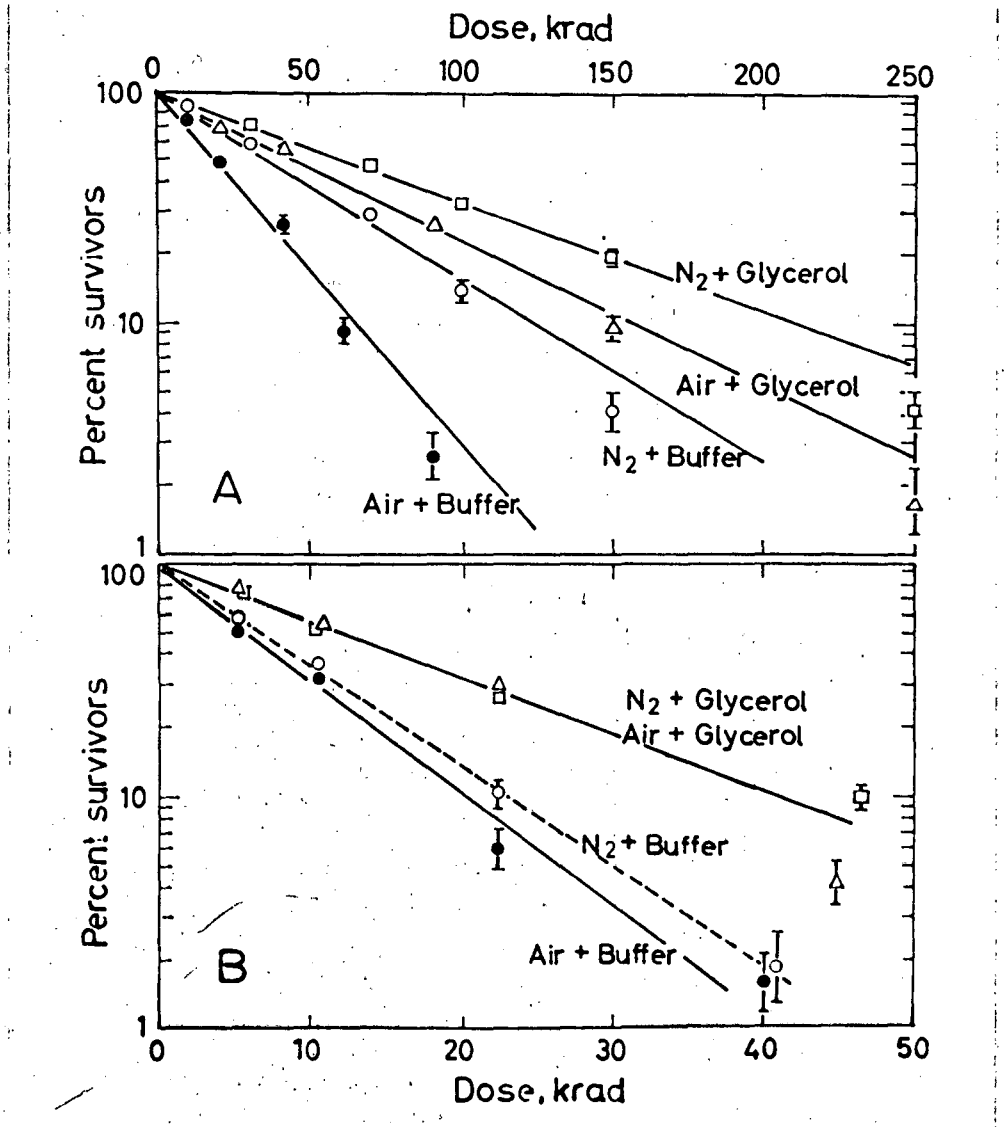
Dose, krad
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Figure 2

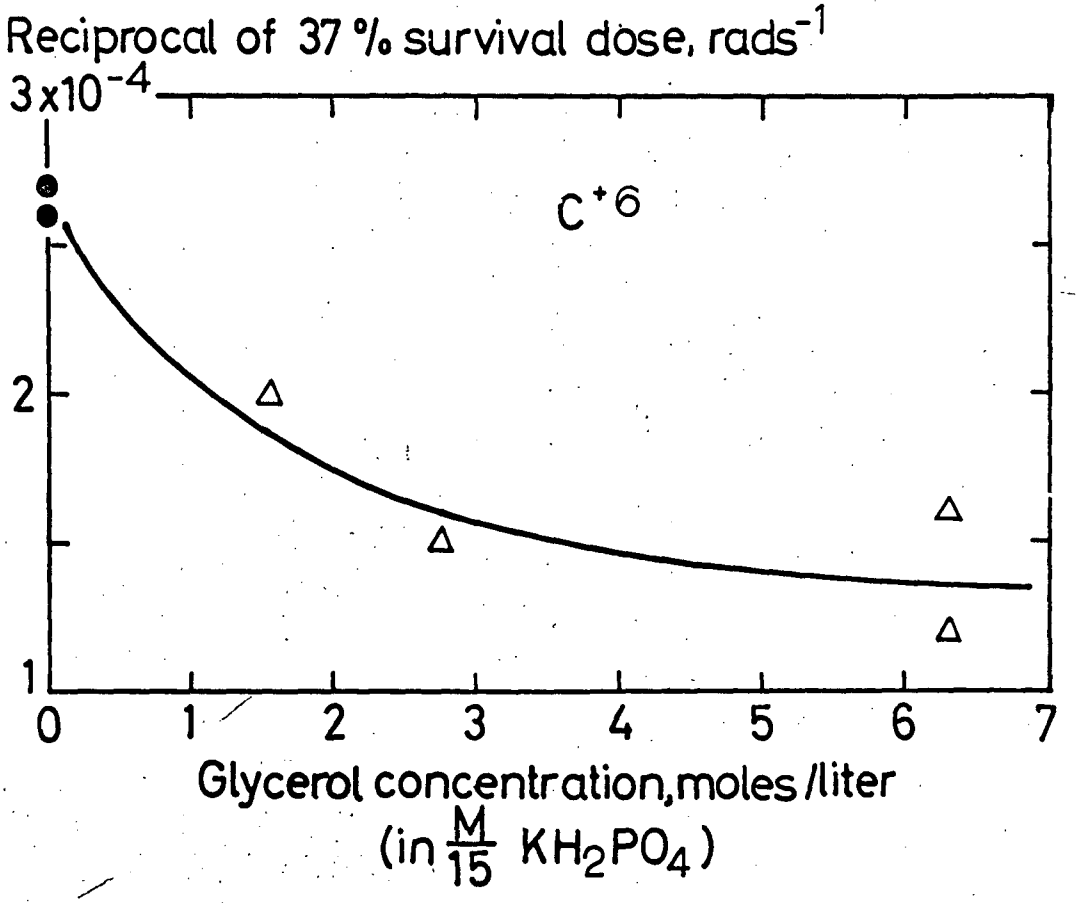
Reciprocal of 37 % survival dose, rads^{-1}
3x10^{-4}
C^{+6}
2
1
0
1
2
3
4
5
6
7
Glycerol concentration, moles/liter
(in $\frac{M}{15}$ KH_2PO_4)

Figure 3

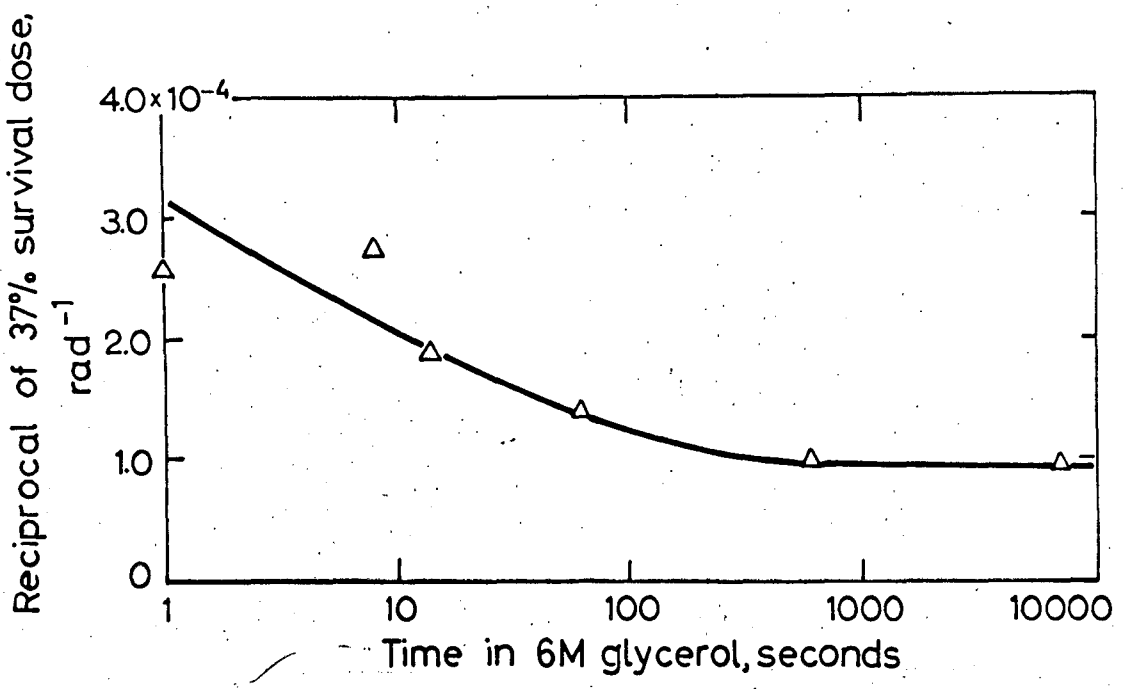
Reciprocal of 37% survival dose, rad^{-1}
4.0 x 10^{-4}
3.0
2.0
1.0
0
1
10
100
1000
10000
Time in 6M glycerol, seconds

Figure 4

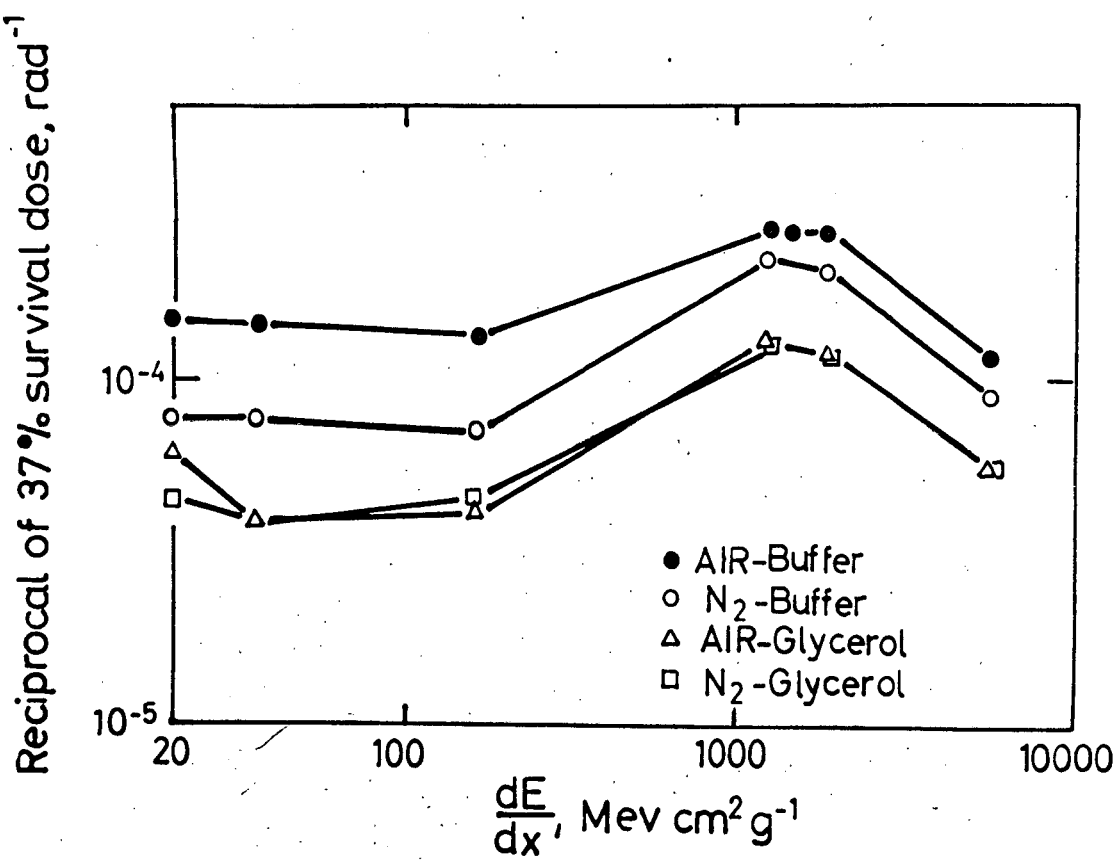
Reciprocal of 37% survival dose, rad^{-1}
10^{-4}
10^{-5}
20
100
1000
10000
$\frac{dE}{dx}$, Mev cm^2 g^{-1}
AIR-Buffer
N_2-Buffer
AIR-Glycerol
N_2-Glycerol

Figure 5

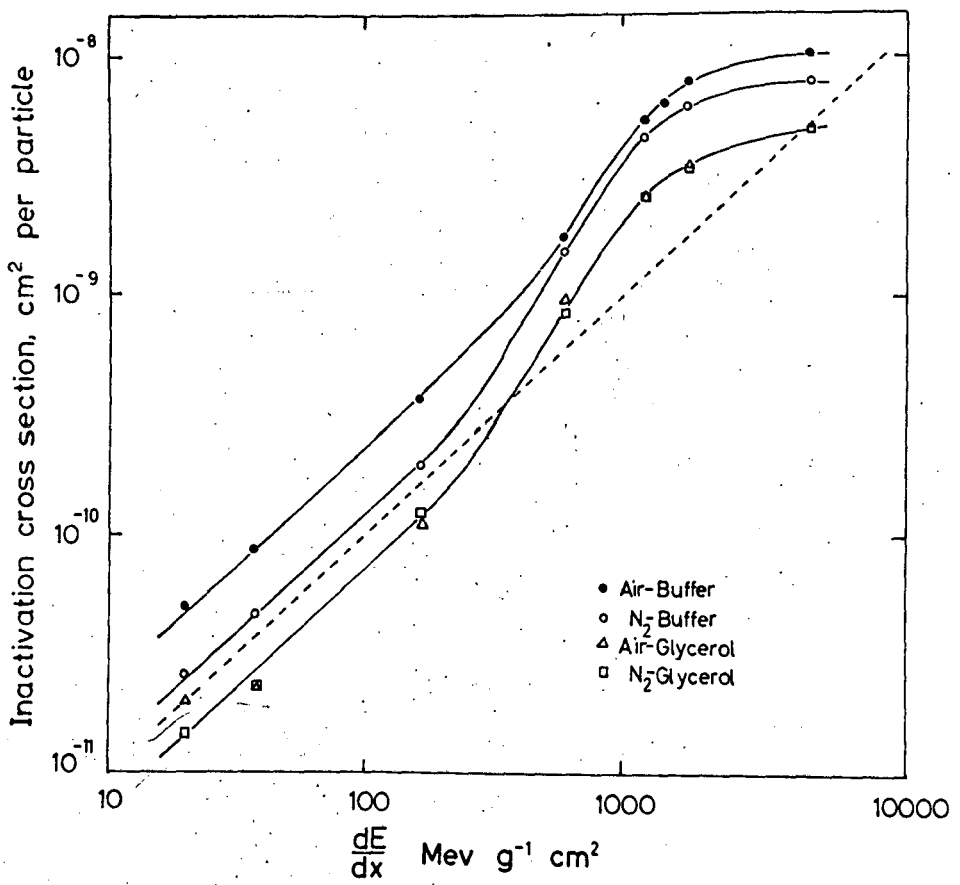
Inactivation cross section, cm² per particle
10⁻⁸
10⁻⁹
10⁻¹⁰
10⁻¹¹
10
100
1000
10000
dE/dx Mev g⁻¹ cm²
Air-Buffer
N₂-Buffer
Air-Glycerol
N₂-Glycerol

Figure 6

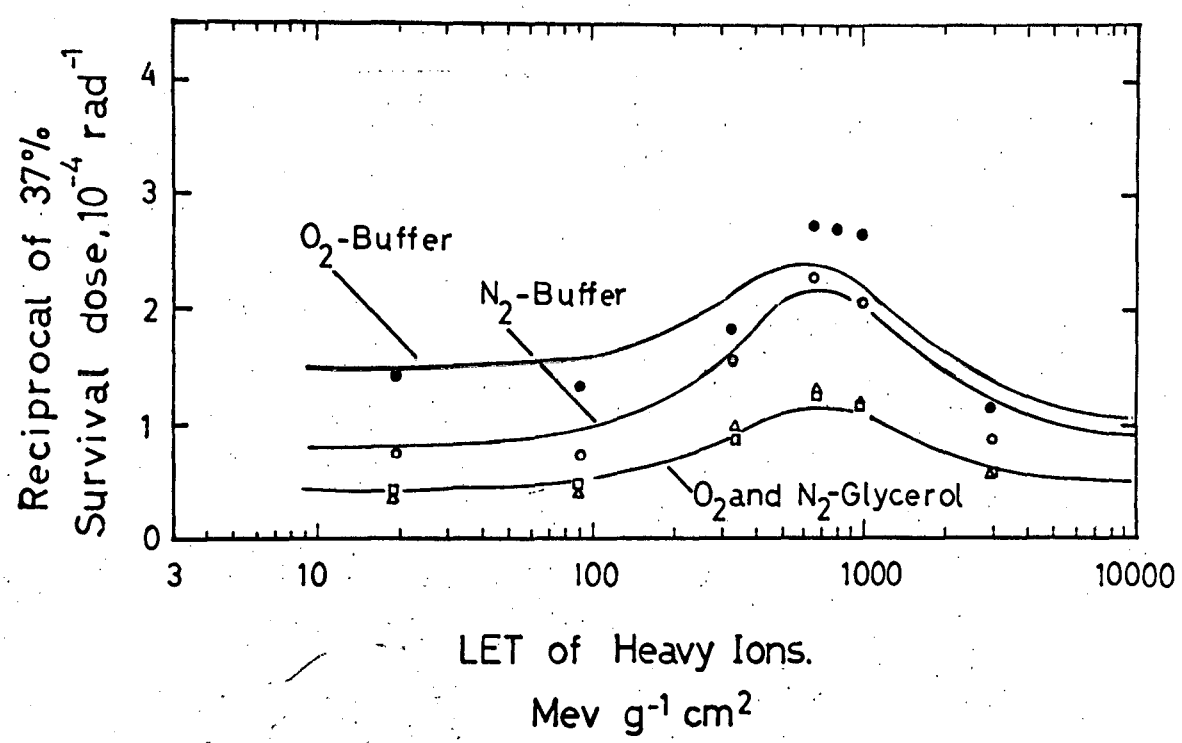
Reciprocal of 37% Survival dose, 10^{-4} rad^{-1}
4
3
2
1
0
O_2-Buffer
N_2-Buffer
O_2 and N_2-Glycerol
3
10
100
1000
10000
LET of Heavy Ions.
Mev g^{-1} cm^2

Figure 7