CONF-770637--1

THE COMBINED EFFECTS OF HYPERTHERMIA AND RADIATION IN CULTURED MAMMALIAN CELLS

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Prepared for

2nd International Symposium
on Cancer Therapy
by Hyperthermia and Radiation
Essen, Federal Republic of Germany
June 2-4, 1977

ARGONNE NATIONAL LABORATORY, ARGONNE, ILLINOIS

operated under contract W-31-109-Eng-38 for the
U. S. ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

To appear in the Proceedings of the 2nd International Symposium on Cancer Therapy by Hyperthermia and Radiation June 2-4, 1977, Essen, Federal Republic of Germany.

The Combined Effects of Hyperthermia and Radiation in Cultured Mammalian Cells

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ABSTRACT

Hyperthermia (temperatures of 39°C or higher) enhances the killing of mammalian cells by ionizing radiation (fission-spectrum neutrons and X-rays). The nature and the magnitude of the enhanced radiation killing varies with temperature and, for a fixed temperature during irradiation, the enhanced lethality varies inversely with dose rate. For temperatures up to 41°C, dose fractionation measurements indicate that hyperthermia inhibits the repair of sublethal damage. At higher temperatures, the expression of potentially lethal damage is enhanced. Since the effect of heat is greatest in cells irradiated during DNA synthesis, the radiation age-response pattern is flattened by hyperthermia.

In addition to the enhanced cell killing described above, three other features of the effect of hyperthermia are important in connection with the radiation treatment of cancer. The first is that heat selectively sensitizes S-phase cells to radiation. The second is that it takes radiation survivors 10-20 hrs after a modest heat treatment to recover their ability to repair sublethal damage. And the third is that hyperthermia reduces the magnitude of the oxygen enhancement ratio. Thus, heat if applied selectively, could significantly increase the margin of damage between tumors and normal tissues.

INTRODUCTION

X-irradiated mammalian cells accumulate sublethal damage before they are lethally affected as evidenced by the presence of a shoulder on the survival curve (1). It is generally found that mammalian cells are able to repair sublethal damage (1,2). The survival curves of mammalian cells subjected to heat also display a shoulder region before exponential killing is obtained (3). There are, however, important differences between cell killing by radiation and heat. The radiation responses of the various lines of mammalian cells are quite similar; e.g., the D_o variation is no greater than 2-fold (1). (D_o is the dose required to reduce survival by a factor of $1/e$ in the exponential region of the survival curve.) Sensitivity to heat varies to a much greater extent. Thus, the heat inactivation rate of Chinese hamster ovary cells (3) was ten times greater than that of pig kidney cells (4). Moreover, the response following radiation or heat varies as a function of the position of the cells in their cell-cycle. These variations, however, are in approximately opposite directions since late S-phase cells are radioresistant (5) while S cells are heat-sensitive (3).

The effect of hypoxia on the response to heat and radiation of mammalian cells is in opposite directions. Hypoxia increases radiation resistance by about a factor of 3. In contrast, under hypoxic conditions heat resistance is decreased (6,7). Other factors drastically increase heat sensitivity while having only minor effects on radiation response. These include environmental acidity (8) and nutritional deficiency (9). Further, cells recover from sublethal heat damage, as they do from sublethal

X-ray damage, but the kinetics is quite different. Sublethal radiation damage is repaired within a few hours (e.g., ref. 2) whereas it takes considerably longer for cells to repair sublethal heat damage (10). An even more striking difference is the transient development of thermal resistance 12-24 hours after an exposure to heat (11).

All the above point to fundamental differences in the way radiation and heat kill mammalian cells. On the molecular level, it is generally agreed that DNA (or DNA plus associated material) is the major target for radiation effects like cell killing and mutagenesis. The target for heat inactivation is not known but most evidence suggests protein denaturation as the critical process (29).

Heat-Enhanced Cell Killing by Radiation

Although an influence of temperature on the radiation response of mammalian cells was suggested already some time ago (e.g., ref. 12), it was not until recently that the first quantitative demonstration that heat enhances mammalian cell killing by radiation was published (13). Since then, the accumulating evidence from various laboraties has pointed to this as a general phenomenon for mammalian cells *in vitro* and *in vivo* (14). Quantitatively the extent to which heat enhances radiation response depends primarily on the temperature and duration of the heat exposure. However, other factors play important roles: the nutritional state of the cells; their position in the cell cycle; the oxygen tension; the exposure sequence; and possibly the local pH.

Figure 1 shows the survival curves of V79 Chinese hamster cells X-irradiated at a dose rate of 3.3 rads/min at various temperatures. Evidently,

temperatures above 37°C lead to progressively steeper curves. The enhanced resistance of the cells when the temperature is raised from 0°C to 37°C is due to repair of sublethal damage during the low dose rate irradiation. As expected, the magnitude of the thermal effect varies inversely with dose rate during simultaneous exposure of the cells to combined hyperthermia and ionizing radiation (15).

The effect of hyperthermia after X-irradiation is shown in Fig. 2. There is enhanced killing when cells are incubated for 2 hr at 40.5°C and 41.5°C compared to 37°C. The effect of 40.5°C is mainly to reduce the shoulder of the survival curve. After 41.5°C, survival is decreased further by the enhanced expression of potentially lethal damage.

Figure 3 shows that hyperthermia also enhances the response of cells irradiated with fast neutrons (31). The effect increases with postirradiation heat treatment and is somewhat similar to that observed with X-rays (compare with Fig. 2). Thus, hyperthermia enhances cell killing even after a radiation the lethal effect of which involves a much reduced accumulation of sublethal damage compared to X-rays.

Heat-Enhanced Killing of Irradiated Synchronized Cells.

Mammalian cells are most resistant to X-rays at close to the same age in the cell cycle where they are most sensitive to heat, i.e., the late S phase (3,5). Therefore, the use of synchronized cells to study the synergism between the two modalities can help to clarify their mode of interaction. For V79 Chinese hamster cells, the X-ray age-response pattern reflects mainly fluctuations in shoulder width (16). Reduction in the

shoulder width of the survival curve of asynchronous cells by hyperthermia therefore should result from a flattening of the age-response pattern. Figure 4 shows that this is the case. Irradiation at either 0°C or 37°C at 12 rads/min results in similar age-response patterns. When cells are irradiated at 42°C at 12 rads/min, the age-response pattern is flattened.

A more detailed analysis requires the determination of survival curves for synchronized cells. This was done with CHO Chinese hamster cells; synergism was observed with S phase cells while no enhanced killing was observed with G_1 cells (10). Figure 5 shows that this is also essentially the case for HeLa cells; the radiosensitivity of late S cells is enhanced much more than that of early G_1 cells by hyperthermia (17).

Inhibition by Hyperthermia of the Recovery from Radiation Damage.

The data presented thus far (Figs. 1,3 and 4) suggest that for mild heat treatments at least part of the enhancement of radiation-induced cell killing is due to the inhibition by heat of recovery from radiation-induced sublethal damage. To examine this possibility, hyperthermia was applied between acute dose fractions. In Fig. 6, when cells were incubated at 37°C between the two X-ray doses, a substantial recovery was observed within 2 hrs. Incubation at 40°C resulted in a lower survival increase. Increasing the inclubation temperature between doses to 41°C for 2 hrs appears to result in a complete suppression of recovery that is irreversible for at least 3 hrs.

This inhibition of recovery by heat may be characterized further by constructing fractionation survival curves as in Fig. 7. During 2.5 hrs at 37°C, a significant shoulder reappears on the fractionation survival

curve. Incubation at 41°C prevents the reappearance of this shoulder. At 41.5°C, in addition to the lack of a shoulder, there is an appreciable downward displacement of the fractionation survival curve. This is due to the ability of hyperthermia to increase the expression of potentially lethal damage (see Fig. 3).

Recovery from the Suppression of Repair of Sublethal Damage by Heat.

As shown in Fig. 6, the inhibitory effect of heat on the repair of sublethal damage appears not to be reversed during 3 hrs after transfer from 41°C to 37°C. Figure 8 shows that it takes surviving cells more than 5 hrs to recover from this inhibition. Immediately after 2 hr at 41.2°C, no shoulder is apparent on the fractionation survival curve. However, if the cells are incubated at 37°C for 5 hr in complete medium before exposure to graded second doses, a small shoulder reappears and this grows in size as the interval at 37°C is increased. By 21 hrs, most of the heat damage responsible for the inhibition of the repair of radiation damage has been lost.

The kinetics of recovery from the heat damage that enhances X-ray damage are shown in Fig. 9. Apparently the repair is slow, compared to repair of X-ray damage; about 9 hrs is required for cells heated in G_1 and up to 20 hr when heat is applied during S. Thus, the kinetics of the loss of the influence of heat on radiation response appear similar for sublethal and potentially lethal damage. However, damage due to heat that interacts with subsequent heat damage to cause thermal killing is probably difference since the repair involved is more rapid (10).

Thermal Radiosensitization of Hypoxic Cells.

An important observation with regard to combined hyperthermia and radiation is the reduction of the oxygen enhancement ratio (OER). This is

shown in Fig. 10 for HeLa cells where 2 hrs at 42°C reduces the OER to about one-half of its normal value (18). Similar results were obtained with bone marrow cells (19) and with spheroids of V79 cells (20). The selective X-ray sensitization of hypoxic cells by heat may be related to the lowering of the pH and nutritional deficiency (8,9). However, these conditions most probably prevail also in the hypoxic foci of solid tumors. Consequently, a lowered OER may apply in the clinical situation when hyperthermia and radiation are combined.

DISCUSSION

Our observation that the X-ray response of cultured V79 Chinese hamster cells is enhanced by hyperthermia (13,15) has been confirmed with a number of other types of cells and hence the enhancement appears to be general (10-15,17-22). In vitro studies have shown that thermal treatment can also increase the killing response following high LET radiation including accelerated helium ions (23) and fast neutrons (31). Synergism is observed not only between hyperthermia and ionizing radiation, but also between hyperthermia and drugs (24,25), UV light, and radioisotopes incorporated into DNA (26). Thus, the potential of hyperthermia as a treatment adjunct (27) is not restricted to radiation therapy but may also apply to chemotherapy.

The data thus far indicate that hyperthermia inhibits the repair of sublethal damage (15,24) and probably also the repair of potentially lethal damage (21,28). Although the molecular mechanisms of these processes are not known, they are probably enzymatically mediated with DNA, or DNA-containing structures, serving as the substrate. Since the evidence supports

protein as the target for heat inactivation (for review see ref. 29), one possible explanation for the synergism is the inactivation by heat of repair enzymes. An alternative, more fully discussed elsewhere (30), is that heat causes changes in chromatin structure that make it less susceptible to repair.

Heat probably enhances the radiation response of mammalian cells by more than one mechanism (26,30). Whatever the mechanisms may be, the cellular responses to combined heat and radiation described here demonstrate two possible advantages for applying this combination in cancer therapy. The first is the selective radiosensitization of the normally radioresistant S phase cells by combining hyperthemia with X-irradiation. The second is that the relatively radioresistant hypoxic cells presumed to be present in tumors may be killed more effectively. Thus, providing that one or both of these advantages can be selectively used against tumor cells, hyperthermia may have some of the advantages of high LET radiations. But in addition, since radiation is given in dose fractions in cancer therapy, advantage also may be taken of the very slow recovery of cells from heat treatment by adjusting the fractionation schedule accordingly.

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FIGURE LEGENDS

Fig. 1. Survival of V79 Chinese hamster cells X-irradiated (250 kVp, 3.3 rads/min) while suspended in DUM-20 at different temperatures, as indicated. (DUM-20 is a medium consisting of 80% buffered saline and 20% Eagles minimum essential medium containing 15% fetal calf serum. DUM-20 does not support division.) Cells irradiated for less than the time required to deliver the highest dose were incubated after irradiation at the same temperature before plating in order that all cells had the same thermal treatment. Data from Ben-Hur *et al.* (15).

Fig. 2. Survival of Chinese hamster cells X-irradiated (as in Fig. 1) and incubated for 2 hr at various temperatures before plating. The dashed line is the survival when plating is immediately after irradiation. Data from Ben-Hur et al. (15).

Fig. 3. Survival of V79 Chinese hamster cells exposed to fission-spectrum neutrons from the JANUS Reactor at the Argonne National Laboratory, U.S.A., immediately followed by hyperthermia. Data from Ngo *et al.* (31).

Fig. 4. Age-response patterns of X-irradiated (12 rads/min) V79 Chinese hamster cells, synchronized with hydroxyurea (HU). the abscissa reprcsents the aging of cells in their growth cycle starting at the G_1-S border (at 0 hrs). Cells were irradiated at the different temperatures as indicated. The doses used reduced the survival of asynchronous cells to the same extent in all three cases. Data from Ben-Hur *et al.* (15).

Fig. 5. Survival curves of early G_1 and late S phase HeLa cells exposed to radiation alone and radiation followed by hyperthermia (43°C, 0.5 hr). The times, in parentheses, indicate when after mitosis cells were treated. Open circles indicate the survival from radiation only and closed circles the survival from the combined treatment. Data (average of two separate experiments) from Kim et al. (17).

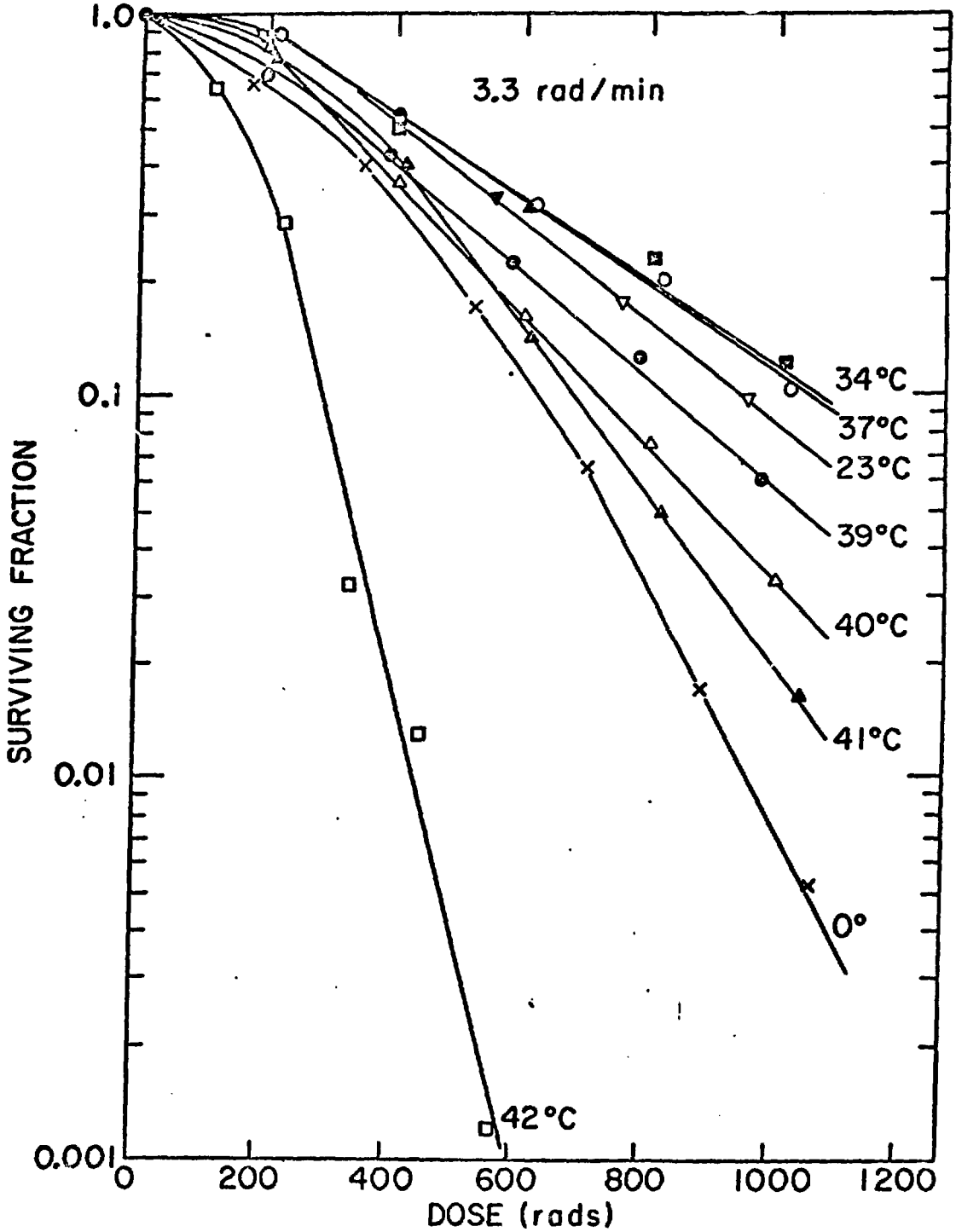
Fig. 6. Two-dose fractionation survival of V79 Chinese hamster cells. (see legend of Fig.1) Cells were incubated at different temperatures in DUM-20/ for various intervals between the doses shown, both of which were delivered at 0°C, dose rate 360 rads/min. Data from Ben-Hur et al. (15).

Fig. 7. Fractionation survival curves of V79 Chinese hamster cells. Cells were exposed to a conditioning dose of 593 rads (see legend of Fig. 1) (0°C, 360 rads/min), incubated in DUM-20/for 2.5 hrs at the temperatures indicated and then exposed to graded second doses. The dashed line is the single-dose survival curve obtained for exposure at 0°C, 360 rads/min. Data from Ben-Hur et al. (15).

Fig. 8. Recovery from inhibition by heat of the repair of X-ray-induced sublethal damage. V79 cells exposed to a first dose of 592 rads (0°C, 360 rads/min) were incubated for 2 hrs at 41.2°C (see legend of Fig. 1). for the first dose in DUM-20 / They were then exposed under the same conditions as/ to graded second doses or after incubation in growth medium at 37°C for various times as indicated. The survival parameters listed are for single cells. Data from Ben-Hur et al. (15).

Fig. 9. Mitotic CHO Chinese hamster cells were plated and then up to 30 hrs later, they were either X-irradiated or were heated and then X-irradiated. The upper curve indicates the survival following 400 rads only. Survival following a heat treatment only of 9 min during G_1 or 7 min during S is indicated by the triangles, the cells being incubated at 37°C before and after the heat treatment. The responses of cells receiving 9 min of heat during G_1 or 7 min of heat during S followed by 400 rads at various times thereafter are also plotted. Labelling-index data and growth curves of cells receiving the various treatments indicate: 1) that for heat treated cells, there was no cell division prior to 35 hrs; 2) that cells heated in G_1 began to enter S at about 20 hrs; and 3) that cells heated in S began to enter G_2 at about 15 hrs. Data from Gerweck *et al.* (10).

Fig. 10. Survival curves of asynchronous HeLa cells irradiated with ^{60}Co gamma rays, under hypoxic (0) or oxic (●) conditions. A. Cells plated immediately after irradiation. The plating efficiency was 65% for both hypoxic and oxic conditions. B. Cells incubated for 2 hr at 42°C immediately after irradiation and then plated at 37°C. The plating efficiency was 50% for oxic controls and 40% for hypoxic controls after heat treatment. Data from Kim *et al.* (18).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0.1
0.01
0.001
SURVIVING FRACTION
3.3 rad/min
34°C
37°C
23°C
39°C
40°C
41°C
0°
42°C
0
200
400
600
800
1000
1200
DOSE (rads)

Fig. 1

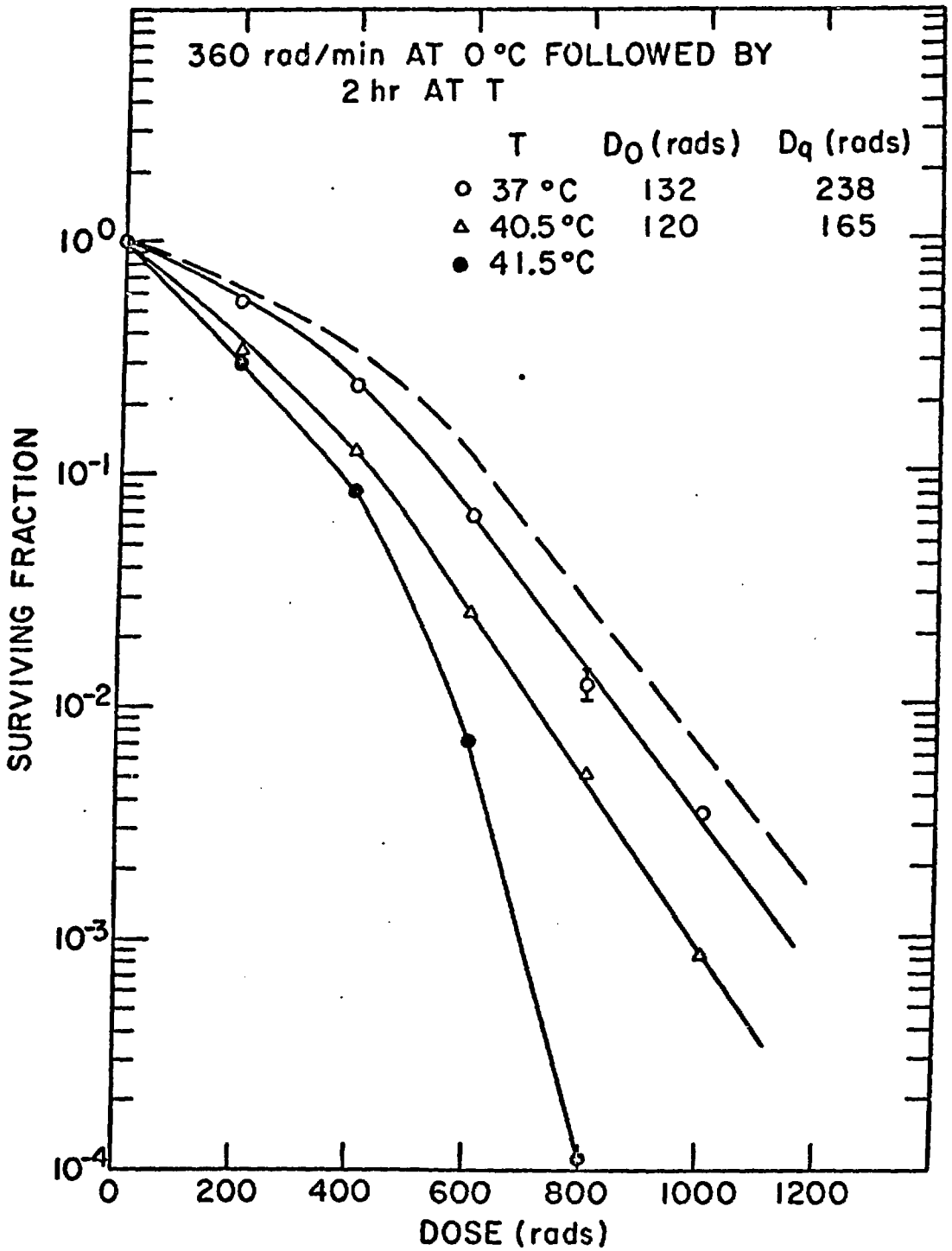

360 rad/min AT 0 °C FOLLOWED BY
2 hr AT T
T
D_0 (rads)
D_q (rads)
37 °C
132
238
40.5 °C
120
165
41.5 °C
SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
200
400
600
800
1000
1200
DOSE (rads)

Fig. 2

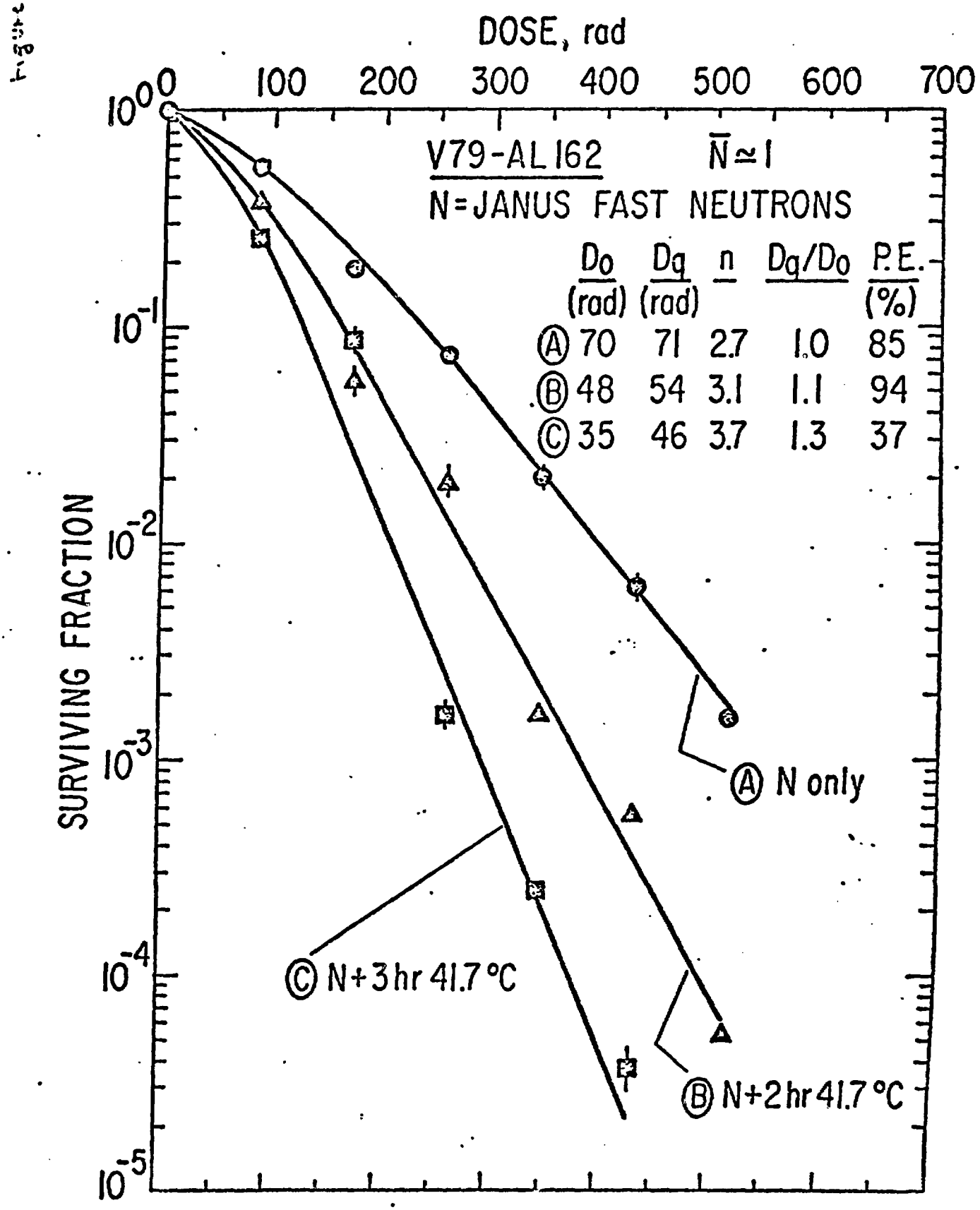
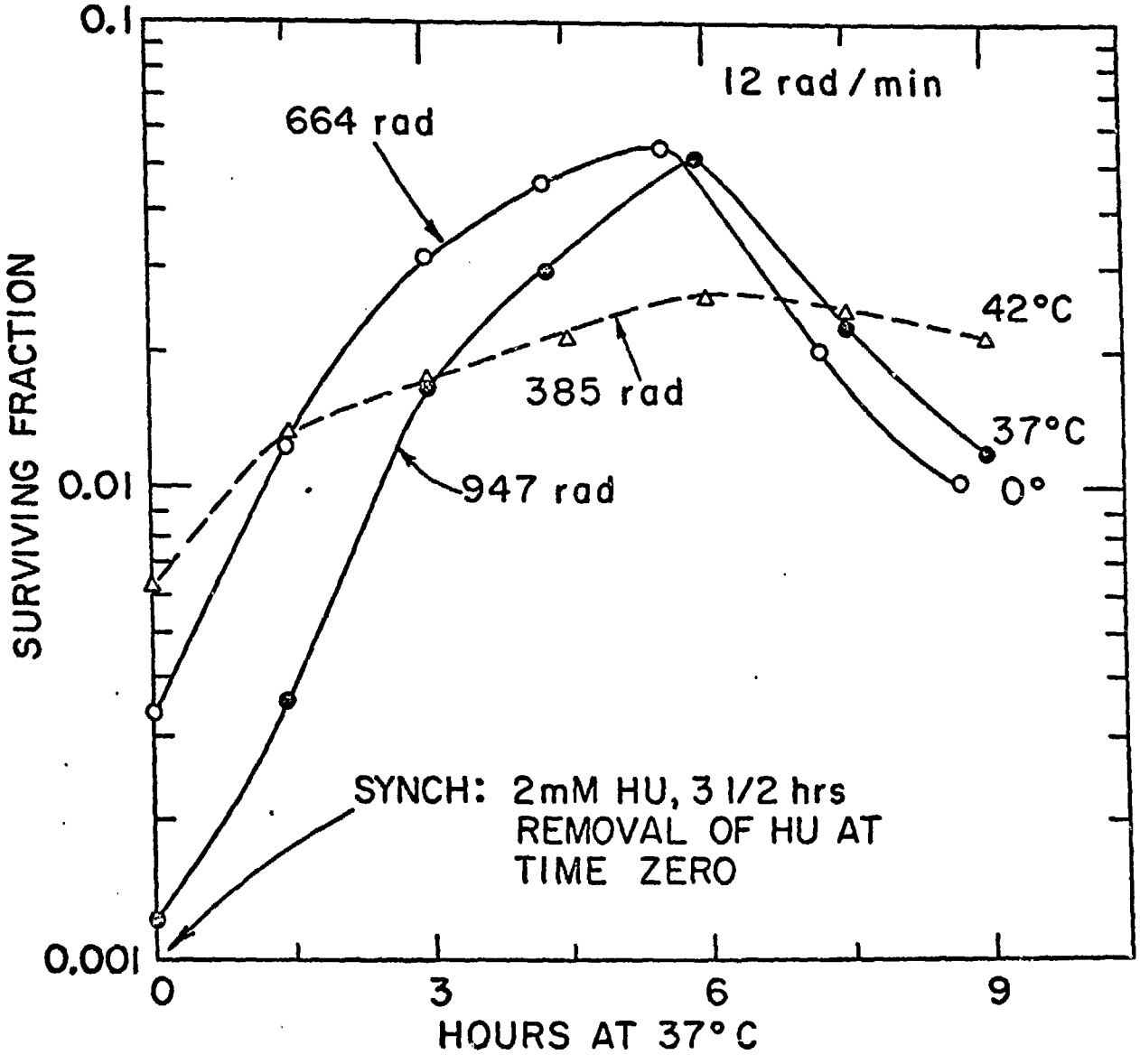

DOSE, rad
0
100
200
300
400
500
600
700
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
SURVIVING FRACTION
V79-AL162 $\bar{N} \simeq 1$
N=JANUS FAST NEUTRONS
Do (rad) Dq (rad) n Dq/Do P.E. (%)
Ⓐ 70 71 2.7 1.0 85
Ⓑ 48 54 3.1 1.1 94
Ⓒ 35 46 3.7 1.3 37
Ⓐ N only
Ⓑ N+2hr 41.7 °C
Ⓒ N+3hr 41.7 °C

Fig. 3


0.1
0.01
0.001
SURVIVING FRACTION
12 rad/min
664 rad
385 rad
947 rad
42°C
37°C
0°
SYNCH: 2mM HU, 3 1/2 hrs
REMOVAL OF HU AT
TIME ZERO
0
3
6
9
HOURS AT 37°C

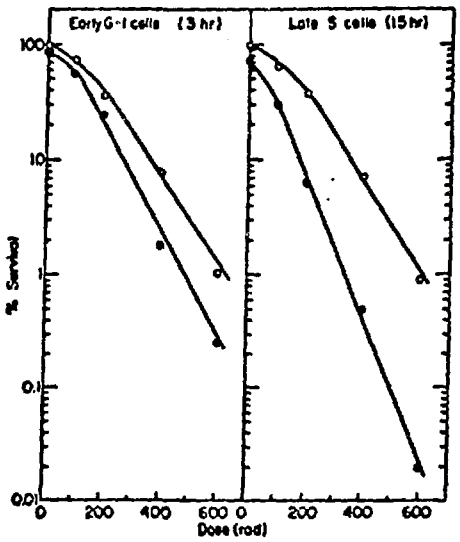

Early G-1 cells (3 hr)
Late S cells (15 hr)
% Survival
100
10
1
0.1
0.01
0
200
400
600
Dose (rad)

Fig 5

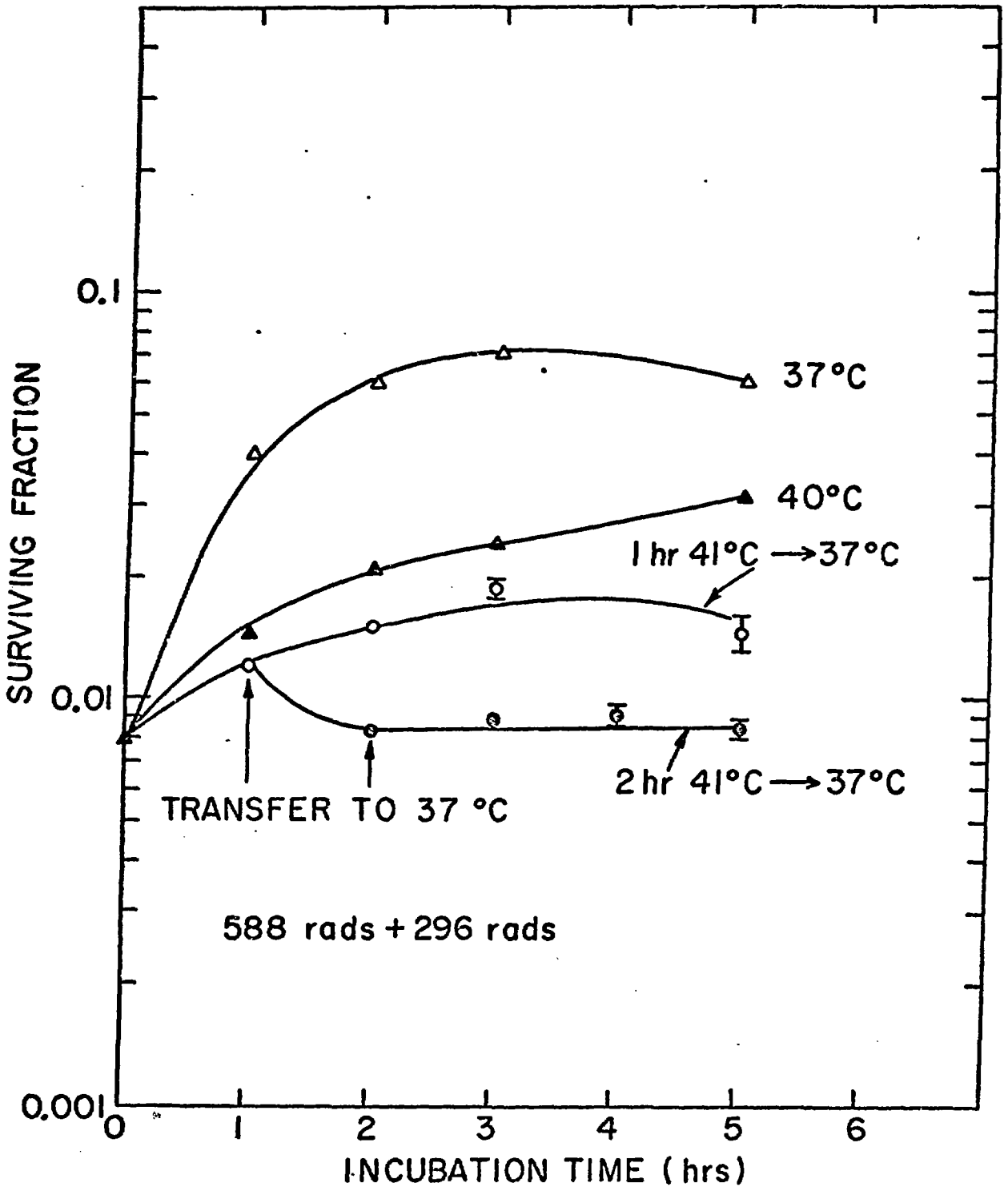

SURVIVING FRACTION
0.1
0.01
0.001
0
1
2
3
4
5
6
INCUBATION TIME (hrs)
37°C
40°C
1 hr 41°C → 37°C
2 hr 41°C → 37°C
TRANSFER TO 37 °C
588 rads + 296 rads

Fig 6

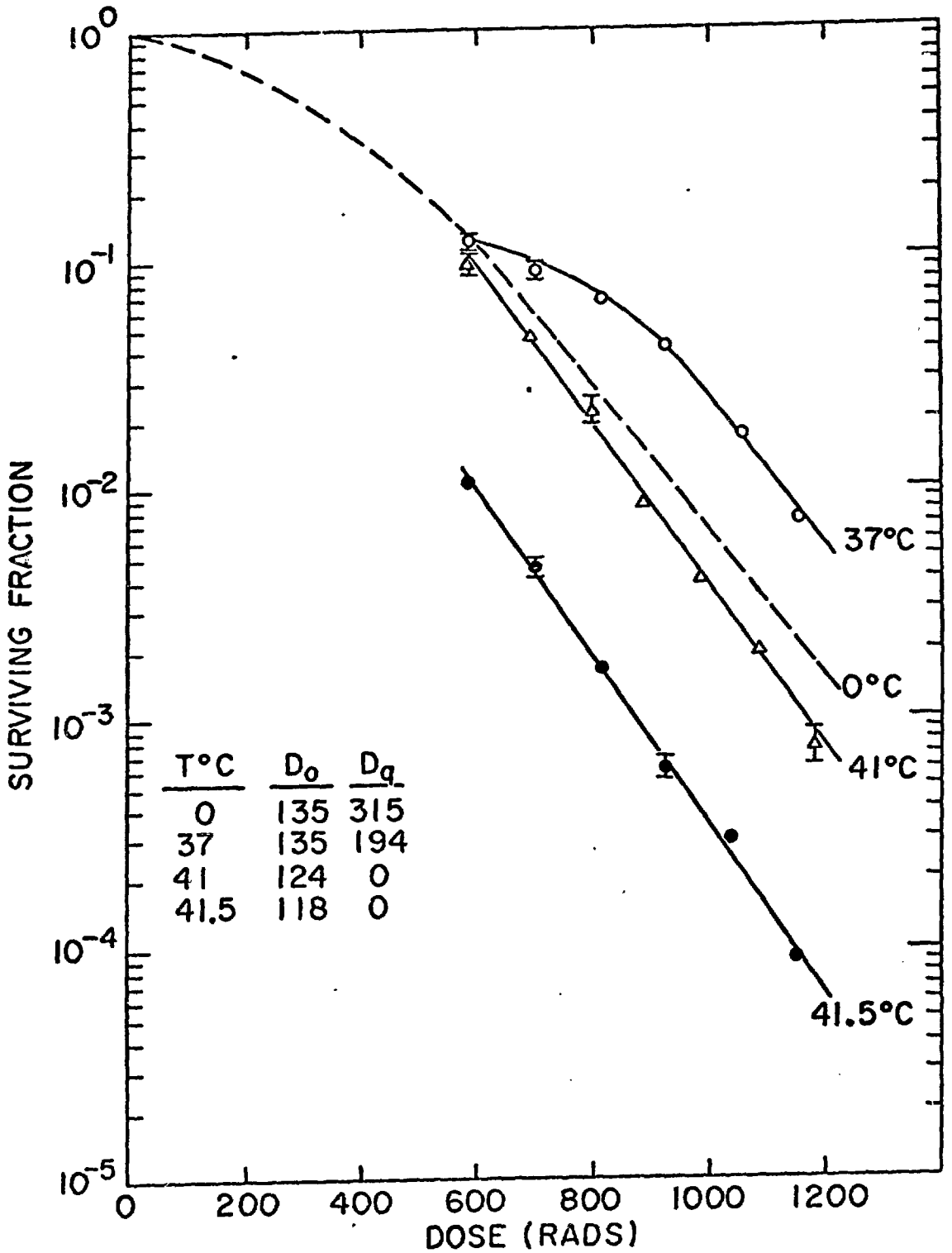

SURVIVING FRACTION
10^0
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
200
400
600
800
1000
1200
DOSE (RADS)
37°C
0°C
41°C
41.5°C
T°C | D_o | D_q
0 | 135 | 315
37 | 135 | 194
41 | 124 | 0
41.5 | 118 | 0

Fig 7

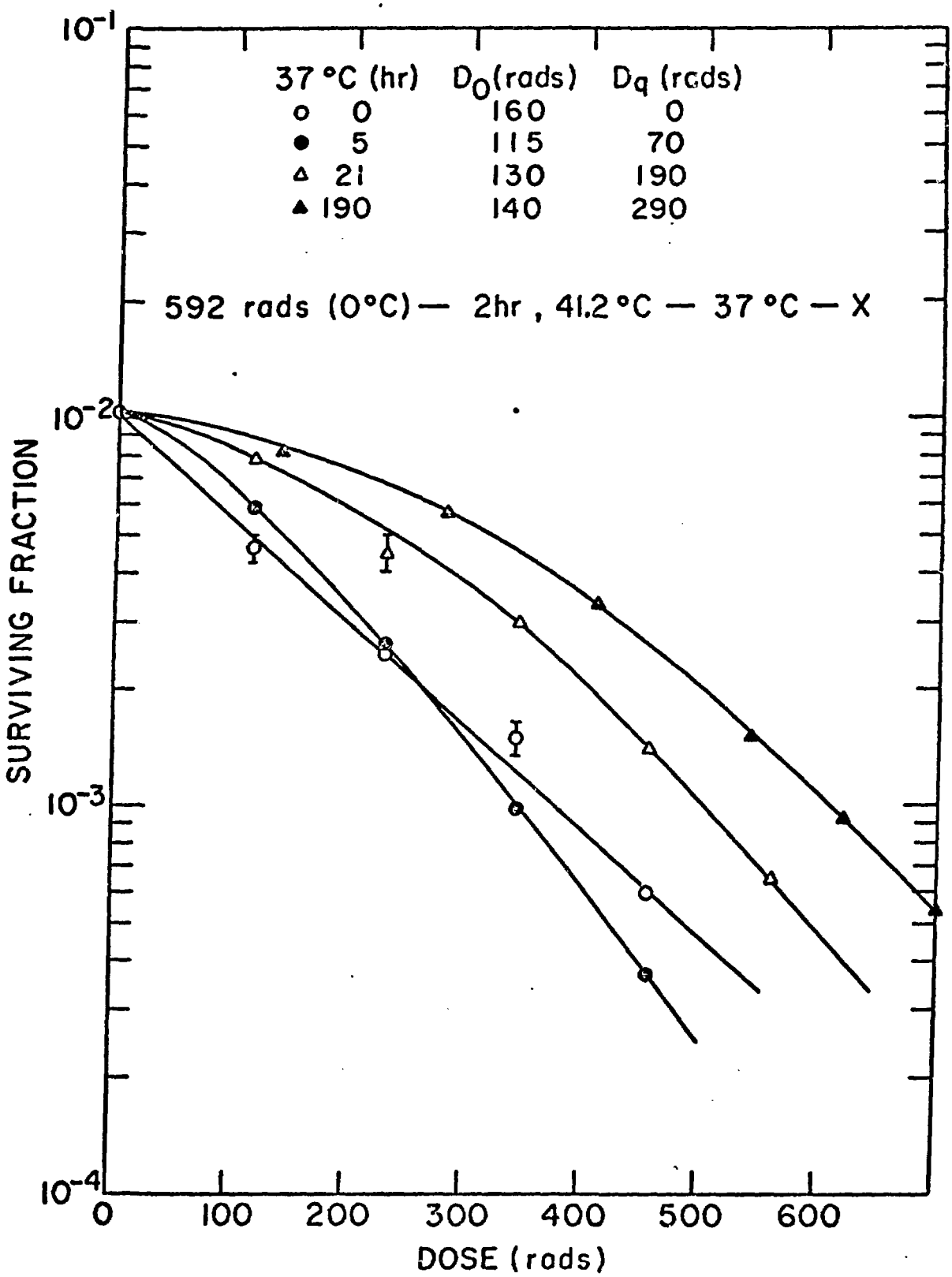

37 °C (hr)
D_0(rads)
D_q (rads)
o 0 160 0
● 5 115 70
△ 21 130 190
▲ 190 140 290
592 rads (0°C) — 2hr , 41.2 °C — 37 °C — X
SURVIVING FRACTION
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
100
200
300
400
500
600
DOSE (rads)

Fig 8

FIGURE 16

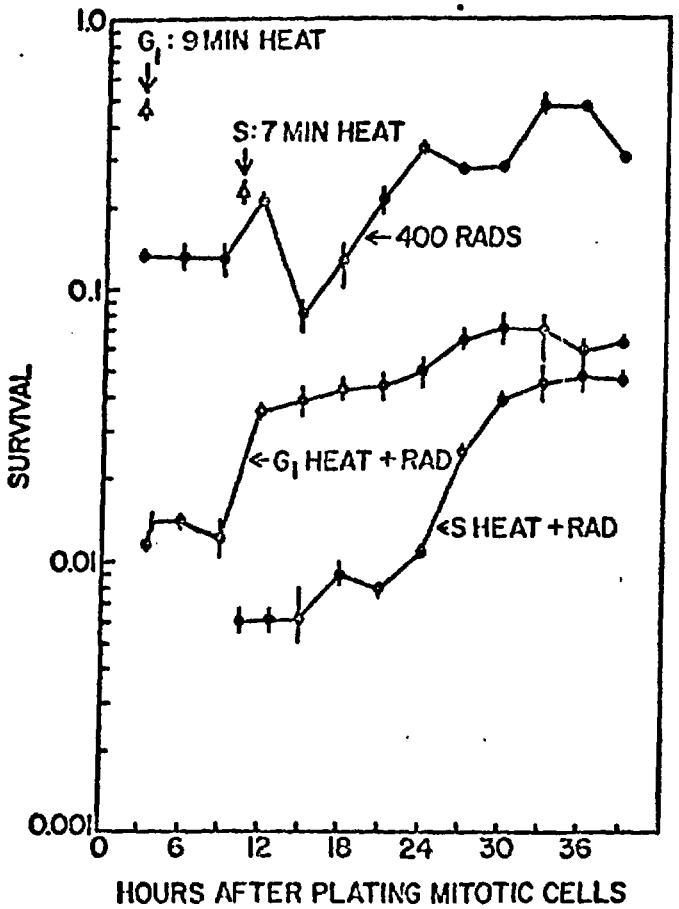
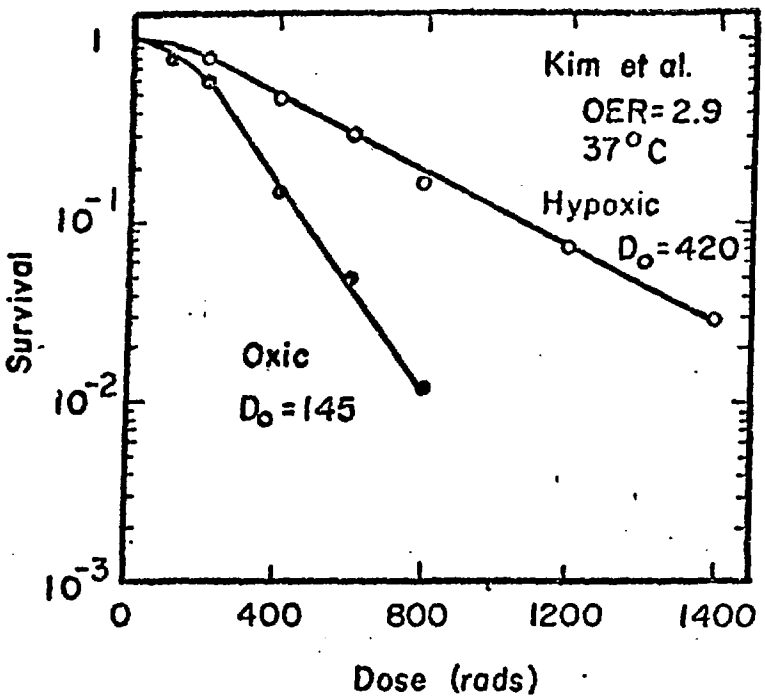


Fig 9

A



F.5=18

B

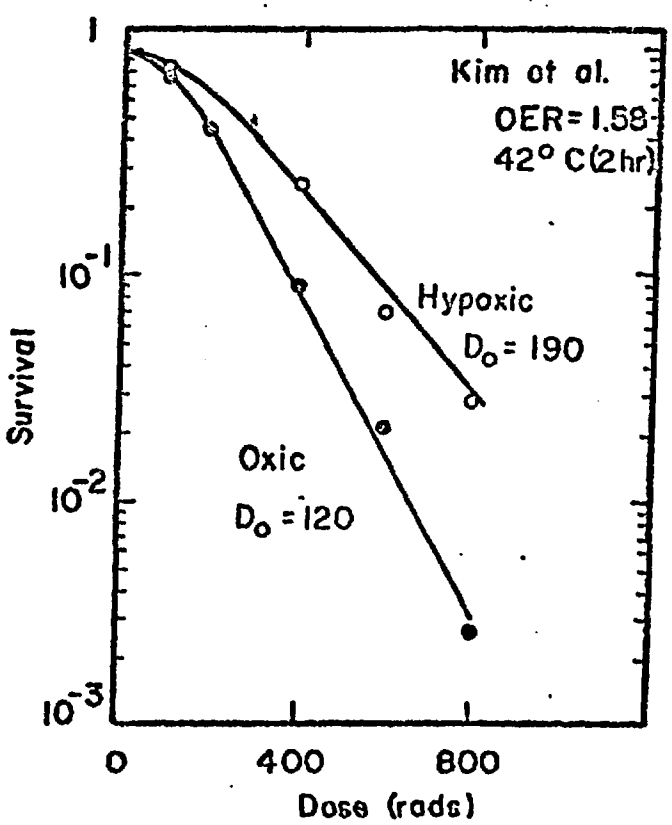


Fig 10