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Cell Recovery Kinetics after Irradiation Combined with Heat

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Abstract

Both successive and simultaneous treatments of gamma rays and high temperature were applied to study the cell survival and recovery kinetics in diploid yeast cells. The extent and rate of the recovery were shown to be lowered with an increased duration of heat treatment (60°Ñ) followed by radiation and with an increase in exposure temperature after the simultaneous thermoradiation action. A quantitative approach describing the recovery process was applied to estimate the probability of recovery per unit time and the irreversible component after the combined thermoradiation action. It was shown that the probability of recovery was independent of the conditions of the thermoradiation action while the irreversible component gradually increased as a function of heat treatment (60°Ñ) duration after the sequential thermoradiation action and as a function of the exposure temperature after the simultaneous thermoradiation action. The rise of the irreversible component was accompanied by an increase in cell killing without postirradiation division. It is concluded that the synergistic interaction of ionizing radiation and hyperthermia in yeast cells is not related to the impairment of the recovery capacity *per se* and may be attributed to the enhanced yield of irreversible damage.

1. Introduction

Hyperthermia is known to enhance the inactivation effect of ionizing radiation in various cellular systems. It is assumed that thermal radiosensitization may be displayed by the inhibition of the repair of sublethal and potentially lethal damage in a cellular level for mammalian [*1-5*] and yeast cells (*6-9*). The observed retardation of the recovery rate after the combined action of hyperthermia and ionizing radiation can be attributed to different reasons: (i) the damage or inhibition of the recovery process itself, when the repair enzymes might be modified in such a manner that they become unable to function; (ii) the increase in the portion of irreversible damage, that could not be repaired at all; (iii) both of these issues. Although the combination of hyperthermia and radiation is of considerable current interest, there have been no reports in the literature on a quantitative estimation of each of these reasons. Thus, the main purpose of this study reported here was to determine whether the synergistic interaction of ionizing radiation and hyperthermia in yeast cells was related to the impairment of the recovery capacity *per se* or to the production of irreversible damage which cannot be repaired. Yeast cells were chosen as a test object for several reasons. First, their recovery has been well studied both in cellular (*10-12*) and molecular (*13-15*) levels. Second, the first idea about the recovery ability in eukaryotic cells has been hypothesized for these cells (*16, 17*). Third, a quantitative approach describing the liquid holding recovery (LHR) of yeast cells was described (*10, 12*) which enables the estimation of the probability of recovery per unit time and the fraction of irreversible damage. Yeast cells have also been used in experiments with the combined action of ionizing radiation and hyperthermia (6-9, *18-21*) but in none of these publications the probability of recovery and the yield of irreversible damage were estimated separately.

2. Materials and Methods

S*trains and Cultivation Condition*

Experiments were carried out using diploid yeast cells of *Saccharomyces ellipsoideus (vini)*, strain Megry 139-B, kindly provided by Dr. V.I. Korogodin (Dubna, Russia). Before irradiation, yeast cells were incubated for 3-5 days at 30°C on a complete nutrient agar layer to its stationary phase of growth. The cells were then washed and resuspended in a 0.07 M phosphate buffer or distilled water to make a stock solution. Final suspensions prepared for irradiation and heat treatment contained approximately 10^6 yeast cells per ml. Such a cell population consisted of single cells with a rather homogeneous cell size distribution.

Irradiation and Heat Treatment

Aliquots with 10^6 yeast cells/ml in a glass tube were exposed to graded doses of γ-radiation. The ^{60}Co γ-ray source was a Gammacell 220 (Atomic Energy of Canada Ltd.). The γ-ray dose-rate, estimated by ferrous sulphate dosimetry, was 10 Gy/min. Hyperthermia was given in a water bath where a desired temperature ±0.2°C was maintained by a constant temperature circulator. The following heating method was used: 0.1 ml of cell suspension at room temperature (about 5×10^7 cells) was placed into 4.9 ml of sterile water or buffer prewarmed to a required temperature in a water bath. For the simultaneous action of hyperthermia and ionizing radiation, the time interval between the placement of the cells into the preheated water and the beginning of exposure was about 0.1-0.3 min, which was significantly less than the total treatment time. At the end of the treatment, the samples were rapidly cooled by cold water.

Liquid Holding Recovery (LHR)

Repair of potentially lethal damage during the LHR is reflected in an increase in the number of vital cells if the cells are kept in an innutritious condition after irradiation for a certain time before they are forced to divide onto a nutrient agar. To observe recovery kinetics, immediately after irradiation, a part of the irradiated cell suspension was placed into conditions (buffer or sterile water, 30°C) promoting the LHR and their colony forming ability was checked again as a function of storage time in a recovery condition (delayed plating).

Survival Assay

Following the treatment, a known number of cells were plated in such a manner that 200-500 colonies would be formed by the surviving yeast cells after 5-7 days of incubation at 30°C. To meet this requirement, 3-50 Petri dishes were used for different experimental points depending on the survival level. The plating efficiency of the untreated cells was 100%. All experimental series were repeated 3-5x. Error bars in all the figures show inter-experimental errors. Dose-effect curves have been drawn by visually fitting the experimental points or using a mathematical multi-hit model. The final results were very similar for both of these cases. The dose effect curves and recovery kinetics were independent of whether the cell suspensions were prepared with 0.07 M phosphate buffer or with distilled water. These results correspond to our previous observations (6, 7) and the data obtained by others (*17, 19-21*).

Inactivation Forms Assay

The information presently available shows that single cells among the inactivated part of the homogeneous population respond differently to the same dose of irradiation. In particular, dying cells can be inactivated either without any division or after one or several reproduction cycles (*22*). In this study, the relative yield of yeast cells inactivated without division was counted microscopically after 24 hours growth at 30°C of cells irradiated at different temperatures. In this case, cells capable of producing a microcolony consisting of 40-50 cells were considered as viable ones. Special investigations have demonstrated that such cells were able to produce a macrocolony visible to the eye after 3-5 days growth.

3. Results

Survival and Recovery Curves

Fig. 1A shows the survival curves of yeast cells irradiated with graded doses of γ-rays without heat or preheated at 60°C for 3, 6, and 12 min. The enhancing effect of hyperthermia on radiation cell killing is observed. Preheating the cells for 3, 6, and 12 min markedly decreased their sensitivity to the subsequent exposure of ionizing radiation. The amount of radiosensitization, i.e. the thermal enhancement ratio estimated by the ratio of slopes constantly increased with the duration of preheating: 1.23, 1.74, and 2.56

for heating during 3, 6, and 12 min. No difference was seen when the sequence order was reversed (data not presented). One can also see that hyperthermia causes a substantial inhibition of the LHR after irradiation. No repair after 60°C hyperthermia applied alone was observed over a period of 80 hr. The degree of inhibition of the LHR by hyperthermia appears to be dependent upon the duration of the treatment. This recovery process is characterized by a constant dose-modifying factor (DMF) over several decades of survival (*19*). In these experiments, the DMF was observed to be 2.30, 1.72, 1.34, and 1.00 for prior heating during 0, 3, 6 and 12 min, respectively. It means that heating for increasing durations at 60°C before irradiation progressively reduced the magnitude of recovery.

The recovery kinetic patterns observed are shown in Fig. 1B. When samples were plated at different times after thermoradiation treatment, the number of viable cells increased as a function of time, reaching a plateau after about 3 days. Heating before irradiation during 3 and 6 min did certainly modify the kinetics by slowing a rate of recovery. Preheating progressively slowed repair and 12 min at 60°C completely inhibited it.

Thus, it is evident that both the extent and the rate of recovery were essentially decreased by exposure to hyperthermia (60°C) prior to ionizing radiation. The observations concerning the survival and the LHR of yeast cells that received a prior hyperthermic treatment confirm the findings of previous works (*19, 23)* and extend their results to show that both the magnitude and the rate of recovery is a function of the duration of hyperthermic treatment.

Studies *in vitro* have shown that the greatest amount of cell killing is obtained when radiation is delivered concurrently with hyperthermic treatment. The results of experiments with the same diploid yeast cells subjected to simultaneous heat-radiation treatment are presented in Fig. 2A. The increase in the exposure temperature in the range of 20-40°C has no influence on the survival (curve 1). It is apparent that the further increase of temperature resulted in an increase in cell radiosensitivity displaying both an increase of the slope and a decrease of the extrapolation number (curves 2-4).

A comparison of the ability of yeast cells in the LHR, carried out at approximately equal levels of survival (i.e. at equal amounts of lethal lesions) is shown in Fig. 2B. Here again, the results indicate that the number of viable cells increased as a function of time, reaching a plateau after about 3 days. The present data suggest that both the rate and the extent of recovery decreased with increasing temperature at which irradiation occurred.

Synergism and Inactivation Forms

For simultaneous thermoradiation treatment, an independent interaction can be determined by SF_C = SF(HT) x SF(RT) and the synergistic interaction is observed if SF_C < SF(HT) x SF(RT), where SF(HT) and SF(RT) stand for the surviving fractions after treatments with hyperthermia and radiation applied alone, respectively. SF_C stands for the surviving fraction after the simultaneous treatment of both modalities. Then the synergistic enhancement ratio can be defined as the ratio of the calculated radiation dose (assuming an independent effect of hyperthermia and radiation) to that observed from the experimental survival curves for the simultaneous thermoradiation action (*6, 7*). Calculated in such a manner is the synergistic enhancement ratio presented in Fig. 3A as a function of the temperature at which the irradiation was delivered. One can see that the synergistic interaction of both modalities takes place only inside a certain temperature range and there is a specific temperature that maximizes the synergy. Similar results have been obtained for other yeast strains exposed to electron beam at high temperatures (9). The overwhelming majority of yeast organisms exposed to ionizing radiation are inactivated and die after at least one cell division cycle. To estimate the yield of cell killing without any postradiation division, equieffective doses of thermoradiation action reducing cell survival to 10% were used. Fig. 3B shows the yield of cell killing without division after simultaneous thermoradiation action on the dependency of temperature at which the irradiation took place. It can be seen that this form of cell inactivation was rare at relatively low exposure temperatures at which no synergistic interaction of the modalities employed was obtained. For more higher temperatures, when the synergistic effect first increased, reached the highest value and then fell with exposure temperature (Fig. 3A), the fraction of cell killing without division was constantly increased (Fig. 3B) reaching 90% at 55°C. Thus, within the temperature range, synergistically enhancing the effect of ionizing radiation, the cell death was gradually transformed from the reproductive to the interphase death. As one can see, the yield of cell killing without postradiation division was identical after heat was applied alone or combined with ionizing radiation.

Evaluation of the LHR Parameters

During the LHR process an amount of primary radiation damage is eliminated resulting in increased cell survival. It can be considered as the reduction in the initial dose D_1 to a certain effective dose $D_{eff}(t)$ which is proportional to the mean number of residual damage, both reparable and irreversible, after

recovery during t hours. Examples of the effective dose estimation are shown in Figures 1 and 2 by the arrows. It was demonstrated (*10, 12*) that the decrease in the effective dose $D_{eff}(t)$ with the recovery time t was fitted to an equation of the form:

$$D_{eff}(t) = D_1[K + (1 - K)\, e^{-bt}], \quad (1)$$

where K is an irreversible component of radiation damage, and b is the recovery constant characterizing the probability of recovery per time unit. In other words, the recovery constant is equal to a fraction of the radiation damage recovering per time unit. Parameters K and b were shown (*10, 12, 24*) to be constant for yeast cell tested over several decades of survival. This equation was used before to fit recovery kinetics of various biological objects irradiated with ionizing radiation alone (*10, 12, 24, 25*) and was never tested for the combined treatments.

The ratio $K(t) = D_{eff}(t) / D_1$ reflects the relative part of the primary radiation damage which has not been repaired during t hours of recovery. If t is sufficiently large (for yeast cells it is about 2-3 days), the recovery curves reach a plateau when the capability of cells to recover is saturated or exhausted. For this moment, we can write

$$K = K(plateau) = D_{eff}(plateau) / D_1. \quad (2)$$

In this expression, $D_{eff}(plateau)$ - the effective dose corresponding to the plateau of the recovery curve and which is proportional to the mean number of irreversible damage. It can be easily shown that

$$e^{-bt} = [D_{eff}(t) - D_{eff}(plateau)] / [D_1 - D_{eff}(plateau)]. \quad (3)$$

Putting $A(t) = [D_{eff}(t) - D_{eff}(plateau)] / [D_1 - D_{eff}(plateau)]$, we have

$$b = -[lnA(t)] / t. \quad (4)$$

In biological terms, $A(t)$ reflects the relative part of the reparable damage that has not been repaired yet after t hours of recovery. Thus, knowing the survival and recovery curves after different conditions of thermoradiation action, one can calculate the corresponding values of $D_{eff}(t)$, $D_{eff}(plateau)$, $K(t)$, K, and b.

Using the results presented in Figs. 1 and 2, we estimated the decrement of the relative part of unrecovered radiation damage $K(t)$ as a function of recovery time for various conditions of thermoradiation action (Fig. 4). These data demonstrate that the values of $K(t)$ fluently decreased reaching a plateau after 2-3 days of recovery both for sequential and simultaneous thermoradiation treatment. One can see the deceleration of the decrement of this parameter by increasing both the duration of prior hyperthermia (Fig. 4A) and the temperature at which the irradiation was delivered (Fig. 4B). It also appeared from these data that the limited quantities of $K(t)$, i.e. the values of irreversible damage $K(plateau)$, increased with increasing the heat load. Fig. 5 shows the increment of the irreversible component as a function of heat exposure (60°C) for the sequential thermoradiation combination (Fig. 5A) and the temperature at which irradiation occurred (Fig. 5B) for simultaneous application of both modalities. It is obvious that the irreversible component progressively increased with thermal load reaching the highest magnitudes by *a prior* heating at 60°C for 12 min and irradiation at 55°C when the LHR is almost completely inhibited due to the lack of reparable damages.

The experimental data presented make possible the calculation of the relative part of the reparable damage $A(t)$, defined by Eqn. 4. The outcome is shown in Fig. 6 both for the sequential (Fig. 6A) and simultaneous (Fig. 6B) thermoradiation action. It is evident that this function decreases exponentially with recovery time and does not depend on the duration of a prior heat treatment or the temperature at which irradiation occurs. Using Eqn. 4 and the results shown in Figure 6, we obtained the recovery constant b = 0.07 hour^{-1} for sequential heat + radiation treatment and b = 0.067 hour^{-1} for simultaneous application of both modalities. It means that about 7% of the residual reparable damage is recovered every hour for both treatments.

4. Discussion

The main purpose of this work was to obtain information as to whether the synergistic interaction of heat and ionizing radiation may be causally related to the impairment or damage of the recovery capacity *per se* or to the production of irreversible damage, which cannot be repaired. The extent and the rate of the recovery were shown to be gradually lowered with an increase both in the duration of heat treatment followed by radiation and the exposure temperature of a simultaneous thermoradiation action. It was

shown in this study that the irreversible component substantially increased as a function of heat treatment (60°Ñ) duration after the sequential thermoradiation action and as a function of the exposure temperature after simultaneous thermoradiation action. For the latter case, the rise of the irreversible component was accompanied by an increase of cell killing without division. To elucidate whether the retardation of the yeast cell recovery occurred only due to the increase of the irreversible component or it was related to the damage of the LHR itself, the recovery constant describing the probability of recovery per unit time was estimated. This parameter appeared to be unchanged (about 0.07 $hour^{-1}$) both with the duration of a prior heat and exposure temperature.. It means that the same part (about 7%) of reparable radiation damage is eliminated for an hour independently of the conditions of thermoradiation action. Hence, the retarded rate of recovery takes place not because the process of the LHR is damaged itself but because of the enhanced yield of irreversible damage. Similar results have been observed for yeast cells simultaneously exposed to UV light and hyperthermia (*26*). It means that some general mechanism of synergistic interaction may be responsible for combined actions of both ionizing radiation and UV light combined with heat.

A bell-shaped curve of synergistic interaction observed here (Fig. 3A) was also obtained for other agents: X-rays and chemical mutagen (1,2-dibromoethane) (*27*), electron radiation and hyperthermia (*7*), ultrasound and hyperthermia (*28*), UV light and hyperthermia (*29*). Here this dependency is presented (Fig. 3A) to compare the synergy and the yield of inactivation forms, which was identical after heat applied alone or combined with ionizing radiation (Fig. 3B). It means that additional irreversible damage is responsible for the synergistic interaction of heat and ionizing radiation resulting in reproductive cell death when ionizing radiation is applied alone.

It would be of interest to speculate on the basis of this study about the mechanism of synergistic interaction of heat and ionizing radiation. As the synergistic effects are not related with the direct damage of recovery process itself, it is not excluded that synergism would be expected to result from some additional lethal lesions arising from the interaction of sublesions induced by both modalities. These sublesions should be considered noneffective after each agent taken alone. This hypothesis was put forward and applied by many authors (*21, 27-30*). Based on the results of this study, the hypothesis could be extended by the supposition that these additional lethal damages are not or less reparable. If it is valid, the retardation or inhibition of the recovery process after combined actions could not be considered as a reason for the synergy, but only as the expected and predicted consequence of such an interaction.

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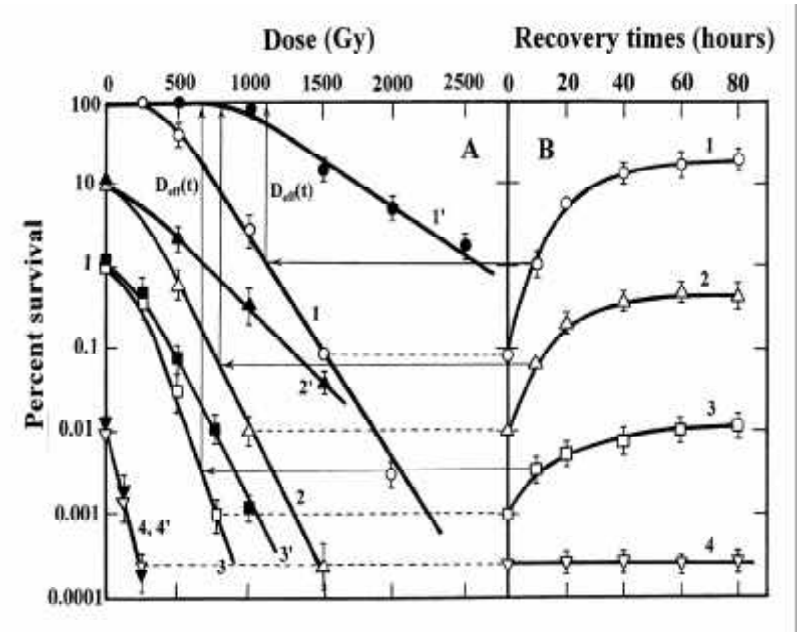

Figure 1. Survival curves (A) and LHR recovery kinetics (B) of diploid yeast cells *Saccharomyces ellipsoideus (vini)*, strain Megry 139-B. A, cells were exposed to graded doses of ionizing radiation alone (curves 1, 1′) or to a combined treatment with a prior heating at 60°C for 3 (curves 2, 2′), 6 (curves 3, 3′), and 12 (curves 4, 4′) min. Cells were plated on nutrient agar immediately after irradiation (curves 1, 2, 3, 4) or after 80 hours of LHR (curves 1′, 2′, 3′, 4′). B, cells were exposed to ionizing radiation alone (curve 1) or to a combined treatment with a prior heating at 60°C for 3 (curves 2), 6 (curves 3), and 12 (curves 4) min. The dotted lines indicate points which were taken for recovery kinetic studying. The arrows indicate examples of the effective dose $D_{eff}(t)$ determination following 10 h of recovery.

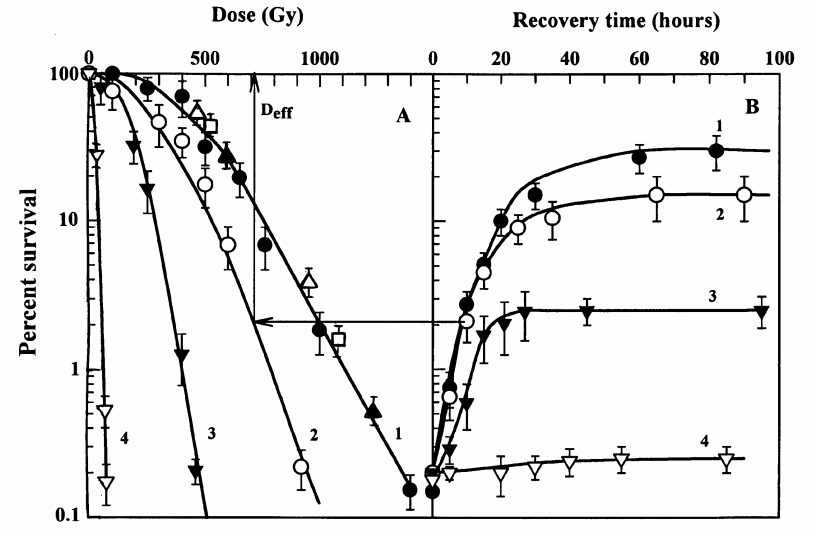

Figure 2. Survival curves (A) and LHR recovery kinetics (B) of diploid yeast cells *Saccharomyces ellipsoideus (vini)* strain Megry 139-B. A, cells were exposed to graded doses of ionizing radiation at various temperature: (curves 1: closed circles - 20°C, open triangles - 30°C, closed triangles - 35°C, open squares - 40°C), 45°C (curves 2), 50°C (curves 3), 55°C (curves 4). The arrow indicates an example of the effective dose $D_{eff}(t)$ determination. Irradiated cells were plated immediately after irradiation (A) or after various recovery times (B, delayed plating).

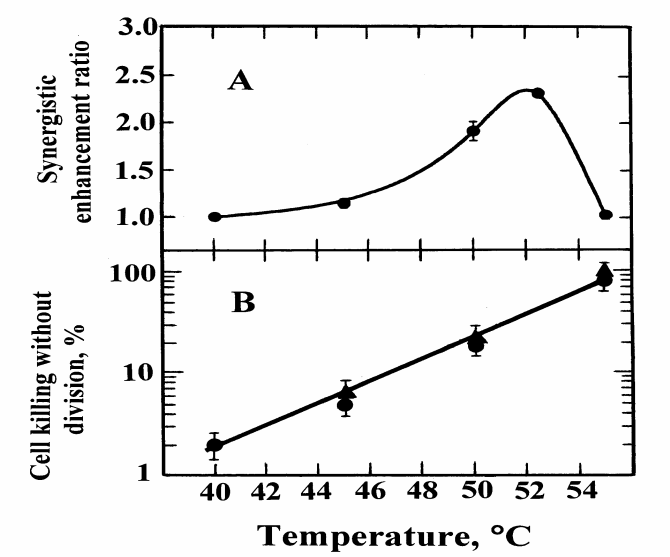

Figure 3. The dependencies of the synergistic enhancement ratio (A) and cell killing without division (B) on the exposure temperature for diploid yeast cells *Saccharomyces ellipsoideus (vini)* strain Megry 139-B irradiated with ionizing radiation at various temperatures (closed circles) or exposed to the high temperatures alone (closed triangles). Data are averaged from three independent experiments. Error bars indicate standard error of the mean. Curves were fitted to the data points by eye.

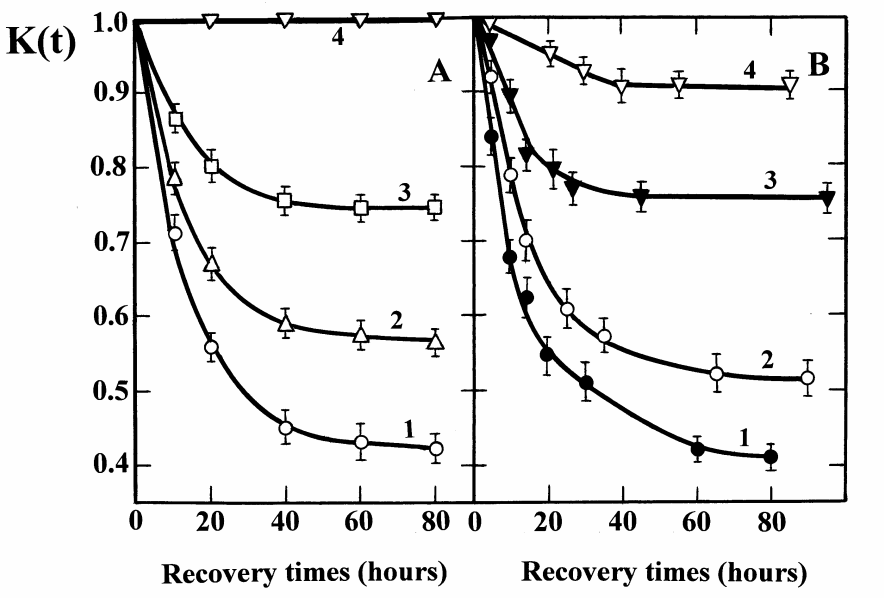

Figure 4. The decrement of the relative part of unrecovered radiation damage $K(t) = D_{eff}(t) / D_1$ as a function of recovery time of diploid yeast cells *Saccharomyces ellipsoideus (vini)* strain Megry 139-B after various conditions of thermoradiation action. A, sequential (heat + ionizing radiation) exposure, a prior heat treatment was 0 (curve 1), 3 (curve 2), 6 (curve 3), and 12 (curve 4) min at 60°C. B, simultaneous thermoradiation action, the irradiation was delivered at the following temperatures: 20°C (curve 1), 45°C (curve 2), 50°C (curve 3), 55°C (curve 4). Data are the mean values calculated from at least three independent experiments.

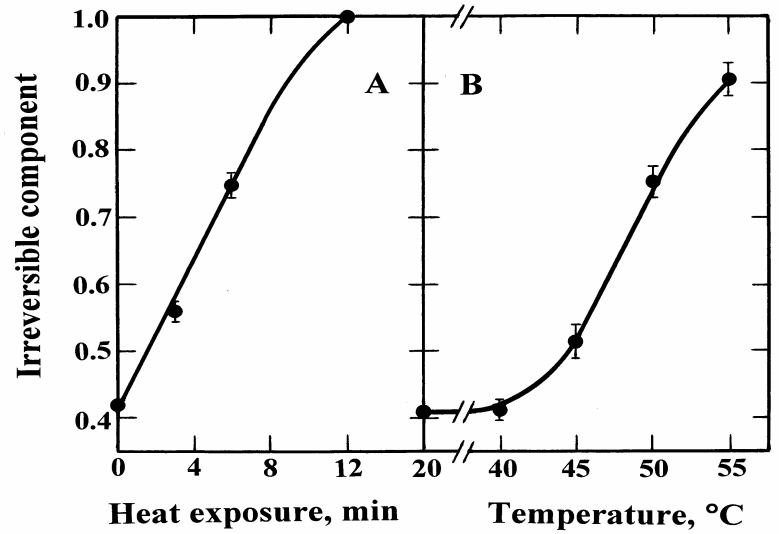

Figure 5. The increment of the relative part of irreversible radiation damage *K(plateau)* as a function of a prior heat exposure (60°C) of the sequential thermoradiation action (A) and as a function of exposure temperature of the simultaneous thermoradiation action (B) in diploid yeast cells *Saccharomyces ellipsoideus (vini)* strain Megry 139-B. Data are the mean values calculated from at least three independent experiments.

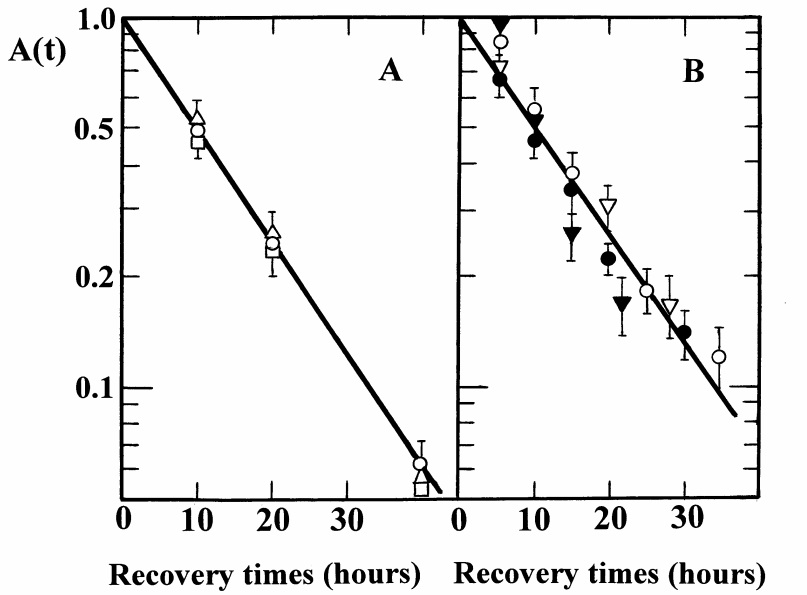

Figure 6. The decrement of the relative part of recovered radiation damage $A(t) = [D_{eff}(t) - D_{eff}(plateau)] / [D_1 - D_{eff}(plateau)]$ as a function of recovery time of diploid yeast cells *Saccharomyces ellipsoideus (vini)* strain Megry 139-B after various conditions of thermoradiation action. A, sequential (heat + ionizing radiation) exposure, a prior heat treatment was 0 (circles), 3 (triangles), and 6 (squares) min at 60°C. B, simultaneous thermoradiation action, the irradiation was delivered at the following temperatures: 20°C (closed circles), 45°C (open circles), 50°C (closed triangles), 55°C (open triangles).