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Repair of DNA Double-Strand Breaks in Irradiated Yeast Cells under Nongrowth Conditions

M. FRANKENBERG-SCHWAGER, D. FRANKENBERG, D. BLÖCHER, AND C. ADAMCZYK

Gesellschaft für Strahlen-und Umweltforschung mbH, München, Institut für Biologie, Abteilung für Biophysikalische Strahlenforschung, D-6000 Frankfurt, Paul-Ehrlich-Str. 20, Germany

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We show that in diploid yeast radiation-induced DNA double-strand breaks can be repaired under nongrowth conditions at 30°C and that liquid holding recovery of colony-forming ability is accompanied by a decrease in the number of double-strand breaks per cell. The kinetics of double-strand break repair under nongrowth conditions depend on the dose applied. For the highest dose used (2400 Gy) repair is completed within about 48 hr. We have verified that double-strand breaks which remain unrepaired after 48 hr are irreparable under nongrowth conditions. The linear relationship between the initial number of double-strand breaks and dose is converted into a quadratic function of irreparable double-strand breaks with and dose during liquid holding treatment of cells.

INTRODUCTION

The possible importance of DNA double-strand breaks (dsb) in cell killing by radiation is suggested by experiments with bacteria (*1, 2*), yeast (*3, 4*), and other eukaryotic cells (*5, 6*). For example, in yeast there is evidence that in mutants deficient in dsb repair one or two unrepaired dsb may result in cell killing (*4, 7*). Repair of dsb has been demonstrated in a variety of prokaryotic and eukaryotic cells when these were kept after irradiation under growth conditions (*3, 4, 8–16*). Holding diploid yeast cells in the nongrowth medium after irradiation produces an increase in surviving fraction (liquid holding recovery, LHR) (*17*). Qualitative evidence has been obtained that dsb repair takes place during liquid holding treatment of these cells (*18*).

In this paper we present data showing that yeast cells are able to perform dsb repair following irradiation when cells are held under nongrowth conditions. Moreover, we have been able to make a quantitative analysis of not only the kinetics of dsb repair under nongrowth conditions, but also the number of reparable and irreparable dsb per cell as a function of radiation dose. We demonstrate that the increase in survival attributable to liquid holding treatment of irradiated cells is accompanied by a decrease in the number of dsb per cell. In yeast both endpoints can be studied in the same radiation dose range, but we have not attempted to establish a causal relationship between the number of irreparable dsb per cell

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and cell killing because we have not yet determined whether breaks, which appear irreparable under nongrowth conditions, might be to some extent reparable when cells are reintroduced into full growth medium, as is the case for cells plated out to measure surviving fraction.

The amount of DNA per yeast chromosome is so small (1.2×10^8–1.5×10^9 g mol^{-1}) (*19–21*) that the neutral sucrose gradient method can be applied to the intact DNA of unirradiated cells, provided that the speed of sedimentation is low enough to avoid a speed effect (*22*). Therefore, dsb induced by doses yielding a high survival can be analyzed. This is in contrast to investigations with mammalian cells where doses used for dsb analysis are invariably much higher than those used for survival studies. Yeast therefore provides a valuable system in which to clarify the possible involvement of dsb in the killing of eukaryotic cells by radiation.

MATERIALS AND METHODS

Yeast strain. Cells of the diploid strain 211*B of *Saccharomyces cerevisiae* in stationary phase were used. This strain is auxotrophic for 2′-deoxythymidine 5′-monophosphate (5′-dTMP), requiring 6–7 μg/ml 5′-dTMP for optimal growth. The strain is a petite mutant, lacking mitochondrial DNA, which allows for specific and efficient labeling of its nuclear DNA. The membrane properties of this mutant seem to be altered; this results in a better uptake of the exogeneously supplied 5′-dTMP (*23*).[1]

Labeling of cells and irradiation conditions. An overnight culture of cells was grown in a double-concentrated medium (4% glucose, 1.3% yeast nitrogen base without amino acids, 0.4% casamino acids, vitamin free) supplemented with 7 μg/ml 5′-dTMP (Boehringer-Mannheim). Cells were diluted into fresh medium; [*methyl*-^{3}H]dTMP (Amersham) was added to give 4 μCi/ml. The culture was rotated for 15 hr at 30°C to yield cells in the stationary phase. The conditions of irradiation with 30 MeV electrons are described elsewhere (*24*).

Treatment of cells under nongrowth conditions. Cells were kept at 30°C for various periods of time after irradiation in nongrowth medium consisting of 67 mmol phosphate buffer, pH 5.0, supplemented with 100 m*M* glucose and 7 μg/ml 5′-dTMP. This medium provided optimal conditions for liquid holding recovery of colony-forming ability.

Survival studies. After appropriate dilution cell suspensions were plated on agar containing 5 g/liter yeast extract, 2 g/liter glucose, 2 g/liter agar, supplemented with 7 μg/ml 5′-dTMP.

DNA sedimentation and quantitative evaluation of the DNA profiles. Cells were protoplasted and lysed on top of a neutral sucrose gradient (5–20%) as described elsewhere (*24*). To avoid a speed effect the released DNA was sedimented at about 9000 rpm in a Beckman ultracentrifuge Model L2-65B at 20°C using the rotor SW 40 Ti. Gradients were fractionated onto glass fiber filters (Whatman GF-B). The filters were dried and the high molecular weight DNA precipitated with ice-cold 6% TCA. The filters were then washed with ethanol and dried. A toluene-based

[1] G. Höltz, *Reparaturkapazität von diploiden Hefezellen gegenüber Röntgenstrahlen.* Thesis, Johann-Wolgang-Goethe Universität, Frankfurt/Main, 1975.

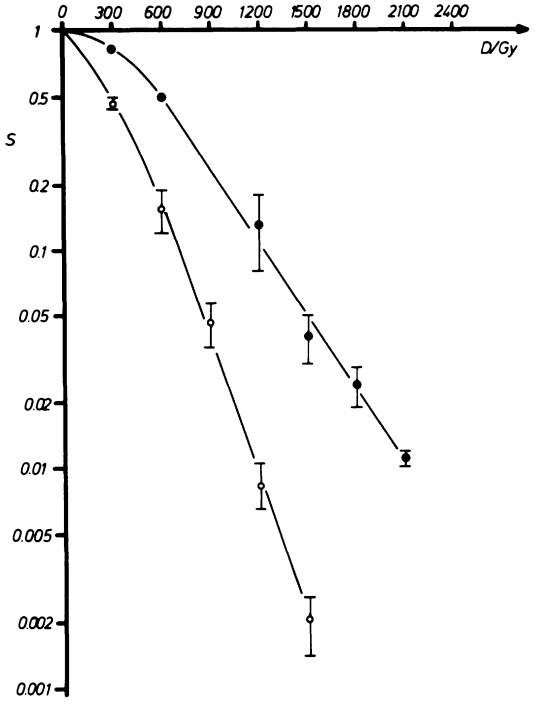

FIG. 1. Effect of 24 hr liquid holding on the survival of irradiated cells. The surviving fraction of cells plated immediately after irradiation (○) and after a subsequent 24-hr liquid holding treatment at 30°C (●) is shown as a function of dose.

scintillation liquid was added to each filter and the radioactivity was determined. The percentage of total radioactivity of DNA in each fraction as a function of the sedimented volume yielded DNA profiles which were used to determine the number of radiation-induced dsb. The average number of dsb per average molecular mass of DNA was calculated by computer simulation of random breakage as applied to the DNA of unirradiated cells and by fitting these calculated curves to the DNA profiles obtained from irradiated cells. Details of this quantitative evaluation of DNA profiles have already been published (*24*). For the calculation of the number of dsb per cell, the molecular mass of DNA of 9.6×10^9 g mol^{-1} for diploid yeast cells was taken (*25*). [See discussion of the values of the molecular mass of DNA in yeast by Lauer and Klotz (*26*) and Lauer *et al.* (*21*).]

RESULTS

Liquid Holding Recovery of Colony-Forming Ability

The increase in colony-forming ability resulting from 24 hr liquid holding treatment at 30°C is demonstrated in Fig. 1. This length of time gave maximum recovery. Longer treatment resulted in a decrease in colony-forming ability. The reason for this decrease at longer times is not known but does not seem to be caused by

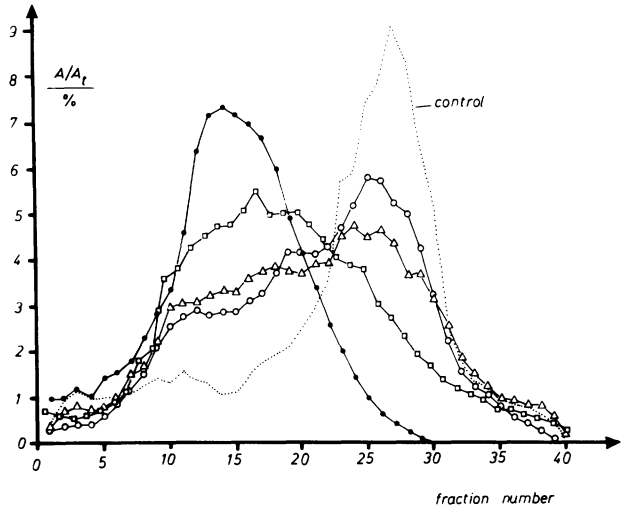

FIG. 2. DNA profiles from irradiated cells after various periods of liquid holding treatment. The percentage of total radioactivity of DNA per fraction (A/A_t) is plotted as a function of the fraction number. The direction of sedimentation is from left to right. DNA profiles are shown from cells analyzed immediately after irradiation with a dose of 1500 Gy (●), and after a subsequent liquid holding period of 3 (□), 16 (△), and 24 hr (○). The dotted line shows the DNA profile of unirradiated cells. The weight average molar mass is 4.8×10^8 g mol^{-1}.

either nutritional deficiencies of the medium or the accumulation of some poison in the liquid holding medium.[2]

Decrease in the Number of Double-Strand Breaks per Cell Due to Liquid Holding Treatment

To examine whether the increase in colony-forming ability resulting from liquid holding treatment of irradiated cells was accompanied by a concomitant decrease in the number of dsb per cell, we performed a parallel dsb analysis on cells immediately after irradiation and subsequent liquid holding treatment for up to 24 hr. Figure 2 shows DNA profiles obtained from cells immediately after irradiation with a dose of 1500 Gy and a subsequent liquid holding period of 3, 16, and 24 hr. In addition, the DNA profile of the unirradiated control cells is shown. The weight average molar mass of the control DNA was 4.8×10^8 g mol^{-1}. With increasing time of liquid holding treatment, a clear shift of the DNA profile of the irradiated cells toward the DNA profile of the unirradiated cells can be observed [Fig. 2 and (*27*)]. This result together with the LHR of colony-forming ability can be interpreted as repair of dsb during liquid holding treatment. Figure 3 shows a summary of the quantitative analyses of DNA profiles from cells analyzed immediately after irradiation and subsequent liquid holding treatment for 24 hr. The number of dsb per cell is plotted as a function of dose. When cells are analyzed for dsb immediately

[2] M. Frankenberg-Schwager and C. Adamczyk, unpublished results.

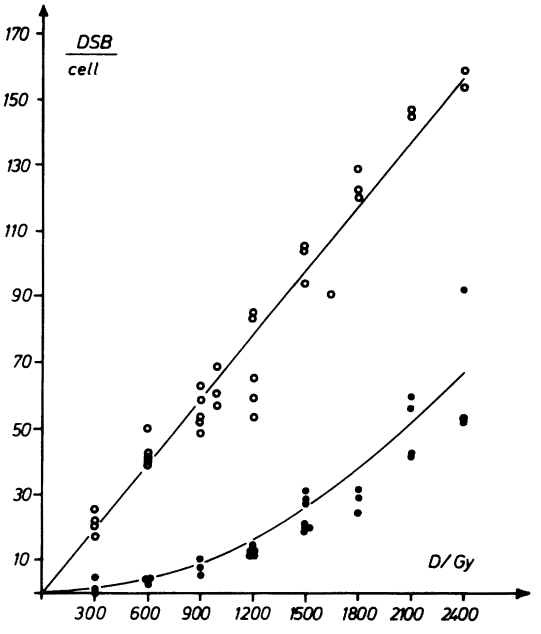

FIG. 3. Effect of 24 hr liquid holding on the number of dsb/cell. The number of dsb/cell is plotted as a function of dose. Cells were analyzed for DNA double-strand breakage immediately after irradiation (○) and after a subsequent liquid holding treatment of 24 hr (●).

after irradiation a linear relationship between dsb and dose is obtained as calculated by regression analysis. The frequency of induction of initial dsb was 0.68×10^{-11} dsb per g/mol per Gy. The number of dsb resulting from two single-strand breaks on opposite strands of the DNA is not significant at the doses used here (*24*). From this result it can be concluded that a DNA dsb is produced by a single energy loss event in irradiated yeast cells (*4, 24*). When irradiated yeast cells are given a liquid holding treatment for 24 hr the number of dsb per cell is drastically decreased and the remaining dsb follow a quadratic relationship with dose. The regression analysis yielded Eq. (1):

$$\text{dsb/cell} = (10.8 \pm 0.4) \times 10^{-6} \times D^2 \times \text{Gy}^{-2}. \quad (1)$$

Kinetics of Double-Strand Break Repair during Liquid Holding Treatment

Figure 4 shows the kinetics of dsb repair during liquid holding treatment of cells which were irradiated with doses of 300, 1500, and 2400 Gy. The time needed for maximum dsb repair depended on the doses cells received. From these results it can be seen that after the higher doses dsb repair was not completed during 24 hr of liquid holding treatment. For example, up to 2400 Gy maximum repair was obtained only after about 48 hr with little or no further repair observed up to 72 hr of liquid holding treatment. Thus in Fig. 3 the number of dsb per cell remaining after a 24-hr treatment still contained a fraction of reparable dsb after the high doses.

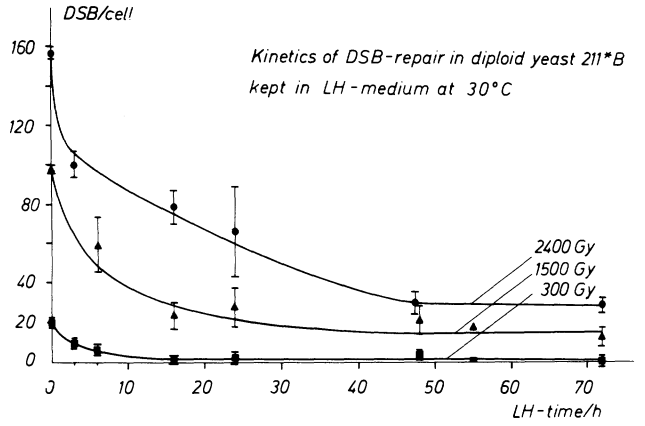

FIG. 4. Kinetics of dsb repair during liquid holding treatment. The number of dsb/cell is plotted as a function of time of liquid holding treatment. Cells were irradiated with a dose of 300 (■), 1500 (▲), or 2400 Gy (●) and analyzed for DNA double-strand breakage after various periods of liquid holding treatment.

Effect of Liquid Holding Medium on Double-Strand Break Repair

Figure 4 shows that with increasing dose an increasing number of dsb per cell remained unrepaired, even after periods of up to 72 hr in nongrowth medium. We wondered whether these dsb were in principle still reparable or whether they remain unrepaired because of the exhaustion of the liquid holding medium. Figure 5 shows that addition of fresh liquid holding medium after a 24-hr liquid holding treatment of irradiated cells did not yield more complete repair of dsb during a subsequent 24-hr liquid holding period than a 72-hr treatment without changing the medium. Also shown in Fig. 5 are the results of an experiment in which cells were kept after irradiation with a dose of 1500 Gy in liquid holding medium deprived of glucose for 24 hr and then analyzed for double-strand breakage. Without glucose in the medium little dsb repair takes place within the 24-hr treatment. Most of the dsb are conserved during such a treatment and can finally be repaired when cells are subjected to appropriate conditions (*32*). Then the cell suspension was split into two samples; fresh liquid holding medium was added to one and "exhausted" liquid holding medium which had already been used for a 24-hr treatment of cells irradiated with 1500 Gy was added to the other. In both media cells repaired dsb to the same extent within the 24-hr period. The results presented in Fig. 5 indicate that cells in nongrowth medium stop repair of dsb although the liquid holding medium apparently still provides optimal conditions for dsb repair. Liquid holding treatments for longer than 72 hr also did not increase the extent of dsb repair; on the contrary, after treatment for 120 hr both irradiated and unirradiated cells showed heavily degraded DNA profiles (results not shown).

Split-Dose Experiments with a Liquid Holding Interval

Using split-dose experiments we have verified that the cells per se were still capable of repairing dsb even after reaching a plateau in dsb repair as shown in the previous kinetic studies. The results are shown in Figs. 6a, b, and c. After

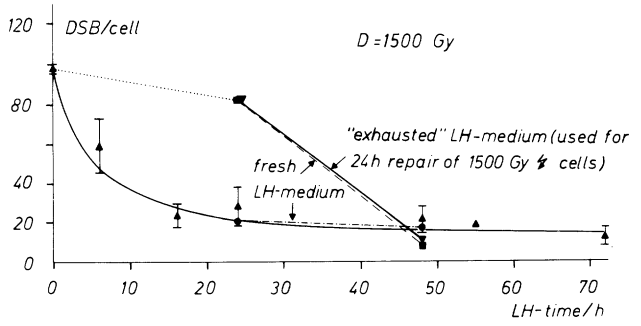

FIG. 5. Effect of liquid holding medium on dsb repair. The number of dsb/cell is plotted as a function of time of liquid holding treatment. The kinetic of dsb repair in cells which have received a dose of 1500 Gy is presented (▲). The results of two experiments are shown: (i) After having received a dose of 1500 Gy cells were allowed to repair dsb in liquid holding medium for 24 hr and then analyzed for remaining dsb. The rest of the cells was resuspended in fresh liquid holding medium, liquid held for another 24 hr, and then analyzed for double-strand breakage (● ·– ·– ·). (ii) Cells were kept after irradiation with a dose of 1500 Gy in liquid holding medium deprived of glucose for 24 hr and then analyzed for double-strand breakage. Without glucose in the liquid holding medium little repair of dsb takes place within the 24-hr treatment (· · · · ·). Then the cell suspension was split into two samples. One sample was resuspended in normal liquid holding medium and repair of dsb allowed for 24 hr (■ – – –). The other sample was resuspended in "exhausted" liquid holding medium which had already been used for a 24-hr liquid holding treatment of cells irradiated with 1500 Gy. After a 24-hr incubation in the "exhausted" medium cells were analyzed for double-strand breakage (▼ ——).

having received a first dose (900, 1500, and 2400 Gy, respectively) cells were allowed a period in nongrowth conditions to perform maximum dsb repair, the time in each case depending on the first dose. Then a second dose was given followed by another liquid holding period in fresh medium sufficiently long to allow maximum repair of those dsb induced by the second dose. All three split-dose studies clearly show that, even though dsb repair levels off after a first dose, cells are still capable of repairing the dsb induced by the second dose with an efficiency equal to that after the first dose. This can be deduced from the final number of dsb per cell, which is about the sum of the unrepaired dsb induced by each of the two doses. Since neither fresh medium nor additional time yielded an increase in dsb repair [although cells were well able to repair dsb even after a 48-hr liquid holding treatment (Fig. 6c)], it seems plausible that the dsb remaining after a 72-hr treatment are those which are irreparable under nongrowth conditions. It is possible, however, that some of these breaks may be repaired when cells are reintroduced into growth conditions.

Relationship between Irreparable dsb and Dose

Figure 7 shows the number of dsb per cell as a function of dose for various periods of liquid holding treatment. The linear relationship between the initial number of dsb per cell and dose was converted into a linear–quadratic relationship after 3 hr of treatment, and into a quadratic relationship after 24, 48, or 72 hr of treatment. The relationship between irreparable dsb per cell and dose after a 72-hr liquid holding period fitting best to the experimental data was calculated by regres-

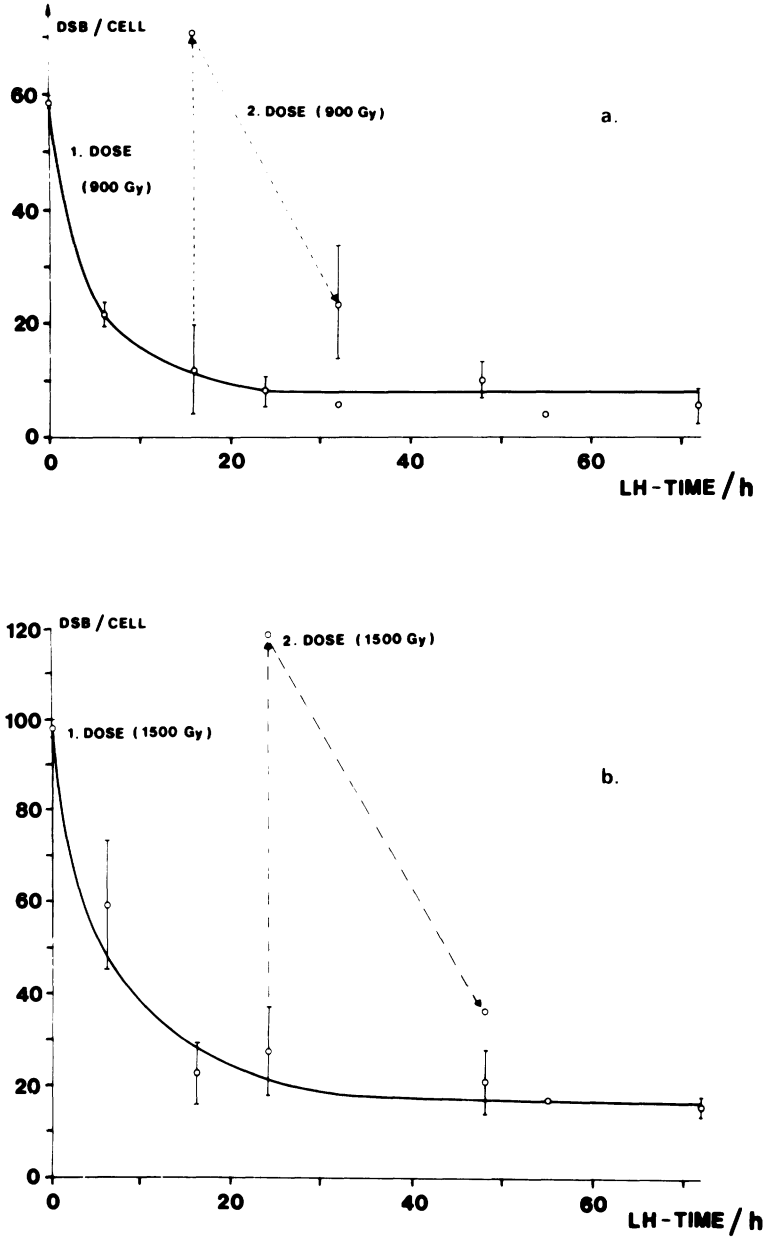

FIG. 6. Split-dose experiments with a liquid holding interval. The number of dsb/cell is plotted as a function of time of liquid holding treatment. After having received a first dose of 900 (a), 1500 (b), and 2400 Gy (c) cells were allowed a period in liquid holding medium to perform maximum repair of dsb, the time in each case depending on the first dose. Then a second dose was given followed by another liquid holding period in fresh medium sufficiently long to allow maximum repair of dsb induced by the second dose.

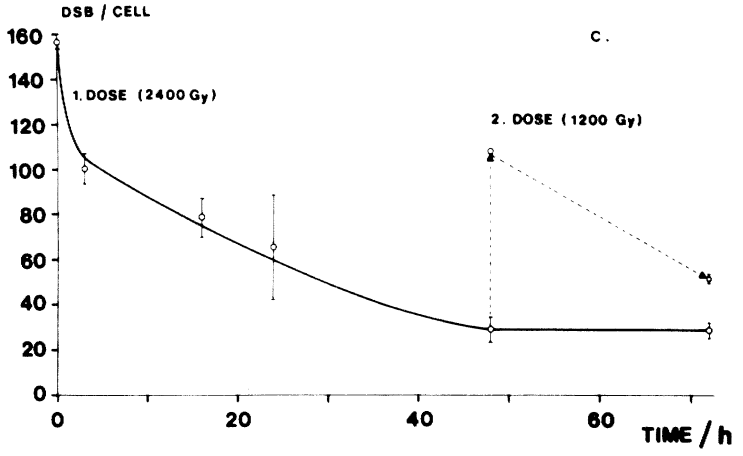

FIG. 6.—*Continued.*

sion analysis yielding Eq. (2):

$$\text{dsb/cell} = (4.94 \pm 0.24) \times 10^{-6} \times D^2 \times \text{Gy}^{-2}. \quad (2)$$

The fit of a linear–quadratic function between irreparable dsb/cell and dose yielded Eq. (3):

$$\text{dsb/cell} = (0.94 \pm 2.54) \times 10^{-3} \times D \times \text{Gy}^{-1} + (4.5 \pm 1.2) \times 10^{-6} \times D^2 \times \text{Gy}^{-2}. \quad (3)$$

The accuracy of our data points does not permit the exclusion of a linear component smaller than 3×10^{-3} dsb/cell $\times D \times \text{Gy}^{-1}$.

DISCUSSION

In contrast to diploid wild type cells, diploid petite cells do not exhibit liquid holding recovery of colony-forming ability when cells are kept after irradiation in distilled water or phosphate buffer at about 30°C in the dark (*28, 29*). However, when the nongrowth medium is supplemented with an appropriate concentration of glucose, irradiated petite cells perform LHR of colony-forming ability [(*30, 31*), Fig. 1]. We show (Fig. 5) that in the absence of glucose from the nongrowth medium dsb repair in diploid petite cells is poor, whereas when glucose is present in the nongrowth medium efficient dsb repair is observed. Irradiated wild type yeast cells display maximum recovery of colony-forming ability after treatment for about 48 to 72 hr. For the mutant used in our studies recovery of colony-forming ability proceeds only up to 24 hr of treatment. Beyond this time no further increase of colony-forming ability is observed, and finally colony-forming ability declines. In spite of the limitation in recovery of colony-forming ability in this strain, data show that cells repair dsb during periods of up to 72 hr (Figs. 4 and 7). Our data

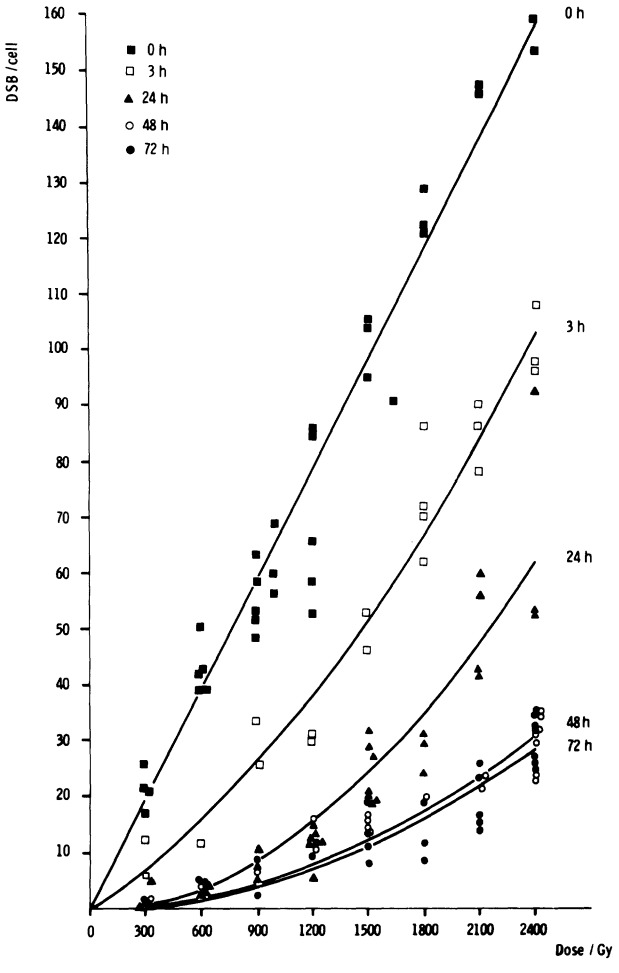

FIG. 7. Repair of dsb under nongrowth conditions. The number of dsb/cell is plotted as a function of dose. Irradiated cells were liquid held for 0 (■), 3 (□), 24 (▲), 48 (○), and 72 hr (●) before analysis of double-strand breakage.

on dsb repair in irradiated cells also demonstrate that after a 24-hr liquid holding treatment not all the reparable dsb are repaired and that cells need longer treatment to repair these dsb. When irradiated cells are plated after 24 hr treatment on nutrient agar as is the case in survival assays, these remaining reparable dsb may be repaired. That this is so has been demonstrated in our preliminary experiments (results not shown) and in the work of Resnick (*32*). Our results do, however, show a clear correlation between an increase in colony-forming ability and a concomitant decrease of the number of dsb per cell due to liquid holding treatment of irradiated cells; this indicates a *possible* causal relationship between dsb and cell killing. Other evidence in favor of a causal relationship between dsb and yeast

cell killing comes from Resnick (*32*), who showed that diploid petite yeast cells exhibit neither liquid holding recovery of colony-forming ability nor repair of dsb when they are kept after irradiation in glucose-free nongrowth medium at 30°C for up to 22 hr. We have confirmed this lack of dsb repair in glucose-free medium (see Fig. 5). Luchnik *et al.* (*18*) demonstrated that haploid irradiated yeast cells exhibit neither LHR of colony-forming ability nor dsb repair during liquid holding treatment. Our data do not allow us to establish a causal relationship between dsb per cell and cell killing because we do not know to what extent dsb left after repair in nongrowth medium will repair when cells are plated on full growth medium. Further experimental evidence is required to prove such a causal relationship.

Our data in Fig. 7 show that maximum dsb repair induced at low doses up to about 600 Gy (surviving fraction 0.15) takes place within a 24-hr liquid holding treatment of cells. With higher doses much longer periods are required for maximum dsb repair. By contrast, when yeast cells are kept under growth conditions after irradiation with a dose of 750 Gy, dsb repair is much more rapid and levels off after about 6 hr when 90–95% of the total dsb have been repaired (*4*). Thus, although radiation-induced dsb are repaired rapidly under growth conditions, they are repaired slowly under nongrowth conditions. In contrast, when cells are plated on nutrient agar immediately after irradiation survival is much lower than that obtained when cells are liquid held in nongrowth medium before plating. Thus it seems that survival of cells is not related simply to the *rate* of dsb repair. Therefore, experiments are in progress to measure the number of irreparable dsb under growth conditions with or without a preceding liquid holding treatment and to relate these results to survival data. Perhaps the rapid repair of dsb leaves either a larger number of unrepaired dsb or a larger number of misrepaired breaks.

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