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Differential Inactivation Analysis of Diploid Yeast Exposed to Radiation of Various LET

I. Computerized Single-Cell Observation and Preliminary Application to X-Ray-Treated *Saccharomyces cerevisiae*

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This series of investigations was designed to observe growth and division of single, diploid yeast cells within the first four generations after irradiation with ionizing radiation. Evidence exists that cell reactions important for the final cell fate occur during this period, and therefore the analysis of cell kinetics and of stationary forms of inactivated cells can be performed. A large number of experiments is necessary to obtain statistically confirmed results of single-cell observation. An automatically steered microphotographic registration device has been developed to facilitate the collection of large numbers of observations. Optical data scanned by a TV camera and digitally stored in a computer are processed by pattern recognizing programs to achieve the correct correlation of newly built cells to existing ones and to deliver a pedigree over four generations of at least eight cells for every irradiated single cell. The pooled data of many pedigrees of this kind allow the analysis of the differential behavior of a total population. From the analysis of X-irradiated cells one can conclude that a single cell that produces at least a microcolony of five cells is eventually able to form a macrocolony and thus can be considered a survivor. That means the division probability of cells to go from generation zero to three corresponds to the survival curve of the colony-forming ability test. Therefore this method is suitable for the differential description of the important phenomenological cell reactions after irradiation.

INTRODUCTION

In radiation biology a physical quantity of primary energy deposition of an ionizing particle is usually correlated with a complex biological end point such as the ability of an irradiated cell to form a macrocolony (cfa). In the results described here, an attempt is made to obtain quantitative data on radiation effects at the level of single-cell reactions, e.g., between the primary effect and the complex biological end point of colony formation. Such data should give a better insight into the mechanisms leading to cell death after irradiation.

Microphotographic registration of irradiated yeast cells has been described in a number of reports. The first investigations illustrated the appearance of abnormal forms of damaged haploid (*1*) and polyploid (*2*) cells. The frequencies of nondividing cells, as well as of those capable of limited division in the progeny of a single irradiated

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cell, have been investigated with respect to dependence on ploidy and different kinds of irradiation although cell pedigrees necessary for kinetic studies were not established (*3*). Pedigree analyses of individual cells have shown that moribund cells appear in clusters among cells that are apparently undamaged (*4, 5*) and that the frequency with which cells initiate "lethal sectors" decreases in successive generations. Different mechanisms have been proposed for this effect (*6–8*). Two types of latent lethal damage can be distinguished, one affecting the irradiated cell and its first division, the other affecting some of the descendants and leading to abortive colonies in later generations. The question arises whether a defined correlation exists between the observed behavior of single cells and the ability to form macrocolonies. Measurements with haploid yeast have been carried out to determine the dose-dependent influence of X rays (*9*), repair (*10*), and other physiological parameters (*11*) on the production of different kinds of damage and the behavior of single cells. However, with diploid yeast pedigree analysis has been used only to study dose-dependent effects of X rays and γ radiation, and there are no reports of kinetic studies or correlations with survival curves.

The aim of our investigations was the quantitative analysis of the inactivation of diploid yeast within the first four generations after irradiation with respect to dose, radiation quality, and repair. It should be possible to use these parameters to determine the time-dependent cell behavior, the distribution of the different forms of abortive cells finally achieved, and a correlation with survival curves of the cfa test.

CINEMATOGRAPHIC REGISTRATION OF SINGLE-CELL GROWTH

An automatic microphotographic method is used for continuous observation of the fate of single cells during a growth period of several generations. It is relatively easy to register yeast cells on solid broth microscopically over a long period using a constant frame (*12*).

For data analysis by computer, however, large numbers of cells and a high accuracy of reproduction are required. These are achieved by a registration assay with the following characteristics:

By the use of a scanning microscope the number of cells at registration start can be varied from 10 to 250 using a series of frames within the slide for recording at a single time step.

The movable microscope table can be reset with an accuracy of ± 1 μm.

The relative coordinates of the cells are changed in a range of only ± 2 μm by rotation or migration within the registration period (e.g., 48 hr), thus allowing the identification of cells in succeeding series.

All cells and their progeny over four generations lie exactly on the focus plane without overlapping each other.

The cells appear as white rings with high background contrast optimal for digitizing by a TV camera and are stored on film (format 24×36 mm^2).

These features are obtained by using a special preparation technique for the slides and optimally adjusting the microscope and its control setup.

Technique and Function of the Microscope Cell Chamber

The microscope cell chamber must support a sufficient supply of cells (up to 10^6) during a period of at least four cell cycles and allow an arrangement of the cells within the focus plane. At the same time the thickness must be limited to achieve high contrast. The chamber is shown in top and sectional side views in Fig. 1. Two plastic rings of different thicknesses (inner ring: 0.1 mm; outer ring: 0.2 mm) are fixed concentrically on a normal microscope slide. After the inner ring is filled with warm fluid nutrient agar, a cover slide (a 0.15-mm-thick glass plate) is put on the outer ring and the agar surface; afterwards the chamber is stored in a refrigerator (4°C). Just before usage the cover slide is removed together with the agar layer. The inner ring is now partially filled with 10 μl cell suspension (10^4 till 10^6 cells) and afterwards agar and slide are carefully replaced. Finally the cover slide is fixed elastically to the carrier slide using vaseline.

The chamber is 0.35 mm thick without the carrier slide. It is best suited for use in the transillumination mode and promises a high-contrast pattern. The cells lie in a plane directly on the carrier glass and are surrounded by a thin fluid suspension layer. The newly budded progeny cells turn and sink because their specific weight (1.15 g/cm^3) is higher than that of the surrounding suspension (1.0 g/cm^3). Thus minicolonies of up to 16 cells are arranged in a monocellular layer as required. The

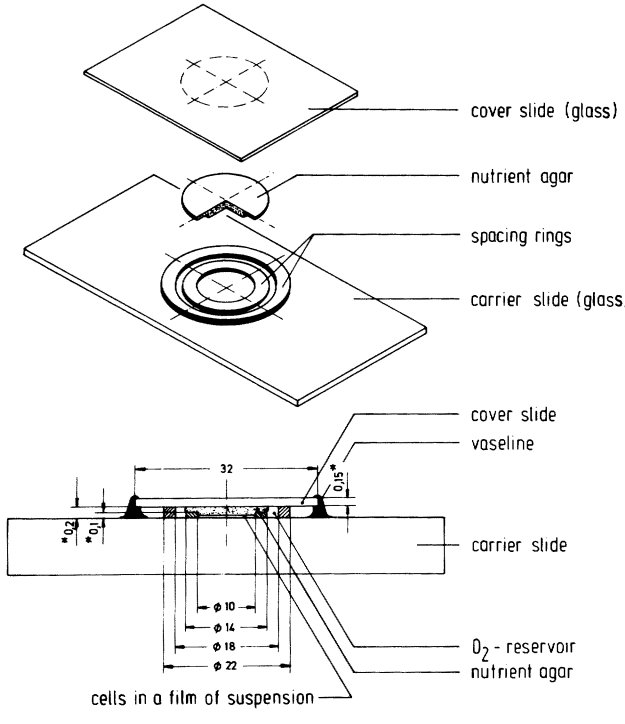

FIG. 1. Microscope cell chamber for yeast cell observation (top) and sectional side view (figured dimension corresponds to millimeters; * indicates drawing out of scale by a factor of 10).

slight pressure of the agar layer on the cells prevents them from migrating and permits a local fixation within a range of ±2 μm.

The cell numbers of microcolonies in dependence on time within postirradiation generations are used as biological parameters. Therefore, the kinetics of cell growth (lag and generation times) in the microchamber must be tested and compared with the corresponding values in nutrient suspension.

To optimize the growth parameters for achieving a constant high growth rate the following values were chosen: The temperature was held constant at 30 ± 0.5°C, where the growth rate is rather insensitive to small fluctuations.[1] The hydrogen-ion concentration change within the microchamber from pH = 6.1 to pH = 4.8 does not influence cell growth because of the measured constancy of the growth rate between pH = 6.5 and 4.5.[2] A sufficient supply of oxygen for at least 10^6 cells [$3 \cdot 10^{-8}$ mole (*13*)] is provided by the special outer ring volume ($1.9 \cdot 10^{-7}$ mole oxygen) of the chamber (Fig. 1). The growth-limiting factor is the concentration of yeast extract of the medium. Seventeen percent of the provided nutrient is needed to produce the maximum cell number.

Experimental proof of undisturbed growth is shown in Fig. 2. A photometrically registered growth curve for cells in suspension is compared with the time-dependent increase of cell number of a population of about 70 single cells. After three cell divisions when the synchronization is approximately decayed the exponential parts of the two growth curves are identical, indicating the same generation times (t_g = 73 ± 2 min; resp. t_g = 73.5 ± 3 min).

Microscope Adjustment and Controlling Setup

Cell patterns with high contrast suitable for digitalization and for further mathematical treatment can be obtained by adjusting the microscope and the steering setup. To increase the contrast without disturbing cell metabolism the path of the light beam of the microscope was changed by lowering the condenser from its normal position, slightly closing the condenser aperture so that only the lower maxima of the diffraction spectrum of the light source enter the plane of the primary picture [Abbe's law of imagery (*14*)], and slightly defocusing (e.g., by 2 μm). The film material used (Agfaortho 25 professional) has a high value of gradation (gamma value γ = 2.5; in comparison with γ = 1 of normal films) and delivers both an additional reinforcement of contrast and a relatively short exposure time, thus avoiding possibly harmful heating of the cells on the microscope slide.

The cell patterns (Fig. 3, magnification ×200), which were derived from a series of six different slide sectors and registered 24 times during an observation time of 6 hr, are clearly visible in black and white pictures of the cells. The cells are fixed locally within a range of ±2 μm and there is no rotation of the whole colonies. The schematic diagram of the technical setup for automatic registration is shown in Fig. 4. A central steering unit controls start and stop of the illumination, the scanning

[1] V. K. Jain, *Vermehrung und Energiestoffwechsel von Hefezellen in der Batch-Kultur.* Thesis, Univ. of Frankfurt, 1970.

[2] H. G. W. Leuenberger, *Kultivierung von Saccharomyces cerevisiae in Kontinuierlicher Kultur.* Thesis, Univ. of Frankfurt, 1971.

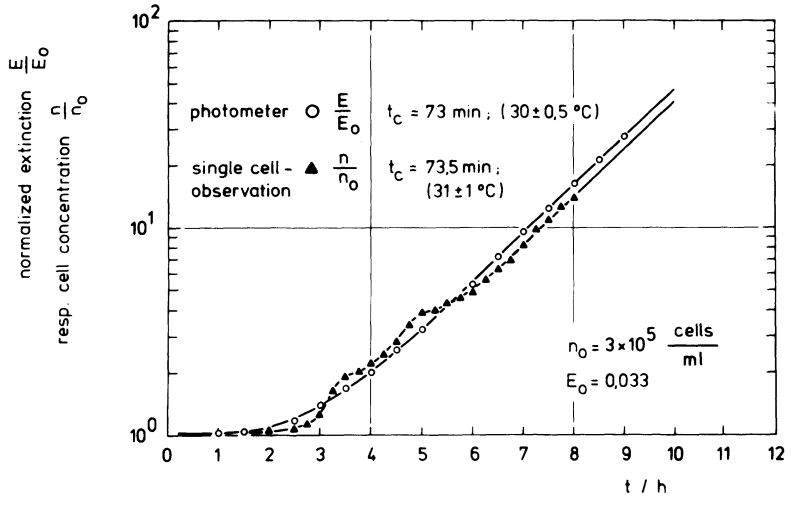

FIG. 2. Comparison of the microscopically registrated growth behavior with the photometrically measured density of cells in suspension.

table, and the camera synchronized with the table. At every observation time (every 15 min with control cells and cells irradiated with low doses, every 60 min after irradiation with high doses) a series of pictures (for example, 6) is registered corresponding to neighboring, not overlapping regions of the slide containing 50 to 100 single cells.

METHOD AND COMPUTER PROGRAM FOR PEDIGREE ANALYSIS

Analysis of the kinetic response and the final fate of damaged cells and their progeny can be performed if pedigrees of a maximum of eight cells are constructed listing the times at which cells are formed, lysed, or misformed.

Method for Automatic Data Processing

The optical information is stored on film (pictures 24 × 36 mm^2). Automatic scanning of these pictures and correlated data recording is performed as follows. A step motor transport steered by a processing computer provides a defined position

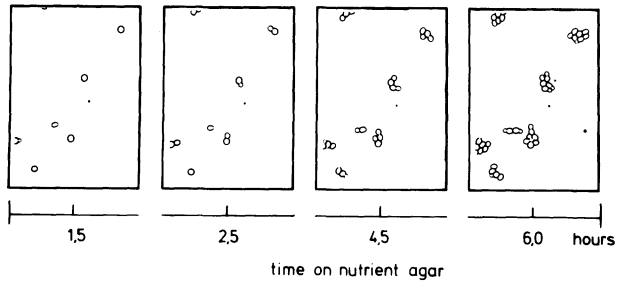

FIG. 3. Yeast growth pattern (magnification ×200) taken from a series of six different slide sectors and registered every 15 min during an observation time of 6 hr.

for reproducible scanning by a TV camera (Leitz-Classimat). The fields of view are translated into black and white using an adjustable gray-level, fed on-line into a digital computer, and stored in a plate storage unit. The change of field of view and the selection of an optimal signal trigger level can be supervised with a TV monitor. The signal trigger level is chosen so that the dark cell rim is characterized by at least three binary digits to distinguish it from background disturbances. The stored data can be processed by a central computer.

Principles of Pattern Recognition and Pedigree Constructing Programs

The digitized information is serially processed with three different programs. The first program eliminates disturbing background signals and generates a data vector for every cell and its descendants including characteristic features and the times of budding (*21*).

The parameters characterizing cells stored as coordinates of the cell vector are: the coordinates of the cell, the smallest and largest value of the cell extension in both the abscissa and the ordinate directions, the area of the inner part of the cell circle (F), the circumference of this inner part (U), the ratio $U^2/4\pi F$ as a form factor, and the time at which a cell is generated.

The biological criterion for a new cell is the production of a bud with a diameter half of the mean for adult cells. The bud appears approximately at the end of the G_1 phase.

The logical criterion used in the pattern processing method for recognition of a new cell is the appearance of an area characterizing the inner part of the dark circle and greater than a presupposed value generated by the program as a mean of many areas.

The task of the second program using the cell data vectors of the program I is the determination of the mother cell from the existing cell assembly for each newly budded cell. In addition, the generation of the new cell is defined and these results (mother cell, generation) are registered as new coordinates of the cell data vector. Finally, a pedigree is constructed from this information for a microcolony of a maximum of eight cells over four generations (from generation 0 to 3).

The third program processes all the pedigrees of the single cells, which are representatives of the population under investigation, and delivers probability distributions of the duration of the G_1 phases and of the cell cycle times as well as their mean values (lag and generation times, respectively) and standard deviations for the analysis of cell kinetics. The distribution of minicolonies of stationary cell numbers after an adequate observation time is used in the analysis of the final fates and forms of cells not able to form colonies (*15*).

APPLICABILITY OF THE METHOD OF SINGLE-CELL OBSERVATION TO X-IRRADIATED YEAST CELLS

The automatic single-cell observation method can recognize the appearance of a progeny of a cell only to the third generation without difficulty. Therefore, whether

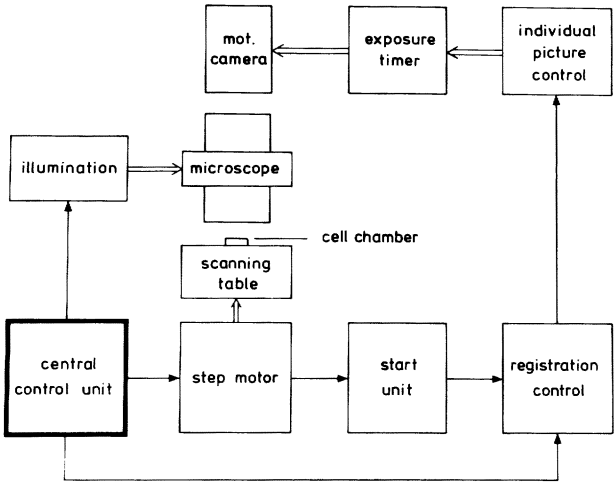

FIG. 4. Schematic diagram of the microscope registration system.

the method can be usefully applied to the irradiated cells depends both on the possibilities for recognizing dose- and repair-dependent effects in irradiated cells and on the availability of stationary forms of damaged cells within the maximum period of microscopic observation. If during this period the damaged cells or their descendants stop dividing then it is possible to discriminate between surviving and nonsurviving cells and a correlation can be made with the survival curves from the cfa-test.

Biological Material and Methods

Diploid yeast cells (*Saccharomyces cerevisiae*) of the homozygous strain No. 211 (*16*) were used. Cells were obtained from a stock culture of stationary state cells on solid nutrient agar (20 g/liter glucose; 20 g/liter Bacto agar; 5 g/liter yeast extract; 4°C) and suspended in phosphate buffer. A synchronized subpopulation (cells in early G_1 phase) was selected by volume sedimentation within a linear density gradient. These cells are characterized by a mean volume $\bar{V} = 30\ \mu m^3$, a mean duration of the first G_1 phase $\bar{t}_{G_1} = 3.1 \pm 0.1$ hr, and a mean cell cycle time $\bar{t}_c = 1.25 \pm 0.04$ hr.

The X radiation used (70 kV, dose rate $\dot{D}_x = 700 \pm 35$ Gy/min; Gesellschaft für Strahlen- und Umweltforschung, Frankfurt) has a track average LET $\bar{L}_{100,T}$ of about 3 keV/μm (*17*) (where the index denotes the cutoff level $\Delta = 100$ eV for δ-ray energies). The absorbed dose was monitored by an ionization chamber calibrated by a chemical dosimeter (*18*). The irradiation was performed using cells in buffer suspension (3 ml; 10^6 cells/ml) stirred under air conditions.

For microscopic observation the chamber was filled with about 10 μl of a buffer suspension containing $4 \cdot 10^5$ to $4 \cdot 10^7$ cells/ml, depending on the degree of damage. The growth of about 50 to 100 cells was registered microphotographically for 10 to 45 hr. An area of 0.36×0.54 mm^2 was selected from the total slide area (78 mm^2)

and scanned. This area was covered by six pictures. Exposures were made at constant intervals. In addition, the colony-forming ability was measured.

Tests were performed threefold as a control before irradiation, immediately after irradiation (immediate plating, IP), and after 48 to 60 hr (delayed plating, DP), during which the cells were suspended in liquid buffer. During the latter period the cells have time to repair radiation damage (Liquid-Holding Reactivation, LHR).

RESULTS AND DISCUSSION

Changes in the ability to grow and divide are very sensitive indicators of radiation damage in irradiated cells because of the participation of important mechanisms of regulation and metabolism in these reactions. By combining all the pedigrees of a population, a differential representation of the division of the total population can be constructed that can be compared with that for unirradiated cells (Fig. 5). The registered zero-generation cells are subdivided into groups $N_i(t_B)$ ($i = 1, 2 \cdots$) to describe the kinetics of the population during the observation time $t_B (0 \leqslant t_B \leqslant t_B^{max})$. $N_i(t_B)$ is a function of time and denotes the number of single cells that have been plated on agar at time $t_B = 0$ and that have produced $(i - 1)$ progeny at time t_B. For instance, the cell group N_i ($i = 4$) denotes the number of zero-generation cells that have generated colonies of four cells; similarly, colonies that consist of more than five cells can be described by the symbol $N_{>5}$. For the graphic description of the population kinetics the portions of cell groups $N_i(t_B)$ are correlated to the zero-generation cell number N_0,

$$n_i(t_B) = \frac{N_i(t_B)}{N_0}, \qquad \text{with } N_0 = N_1(t_B = 0) = \text{constant}, \tag{1}$$

and indicated as relative cell group n_i. The time-dependent pattern of the various relative cell groups is called the "population spectrum" and represents the distribution of all cells of the population with respect to time.

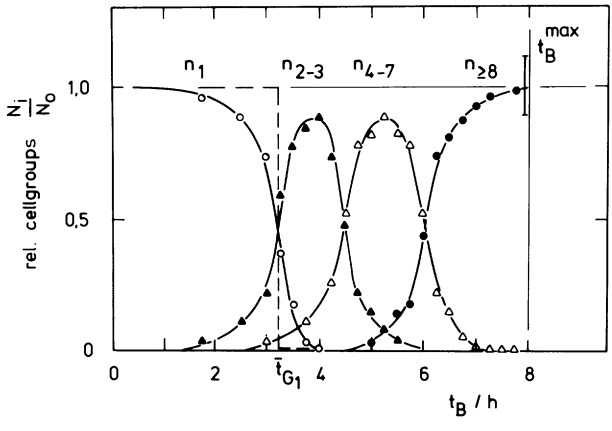

FIG. 5. Time-dependent behavior of the relative portion of cell groups $n_i(t_B) = N_i(t_B)/N_0$ of unirradiated yeast cells growing for the time t_B on nutrient agar [N_i is the fraction of single cells N_0 having produced at least $(i - 1)$ progeny].

The number of single cells starting their growth at time $t_B = 0$ will decrease due to the formation of double cells, which corresponds to a step function (indicated as a dashed line in Fig. 5) if the population is synchronized optimally. After the generation time t_{g2} the double cells will become colonies of four cells and so on. Due to the different G_1 phases and the different division delays in later generations the time course becomes partly desynchronized. For instance, after 4 hr both double cells and the first four cells groups can be observed. Therefore the n_{2-3} group does not reach the maximum value of 1. After 8 hr, however, all the cells have grown to colonies of eight or more cells.

The radiation damage to cells causes time shifts, loss of symmetry, and the occurrence of cell groups containing less than eight cells at the end of the observation period. These effects are demonstrated in Figs. 6 and 7, which show the time-dependent behavior of single cells influenced by two different X-ray doses after both immediate plating (IP) and delayed plating (DP).

In controls the n_{2-3} curves and n_{4-7} curves sink symmetrically after passing through the maximum (Fig. 5). In irradiated populations this is not the case. Generally, the

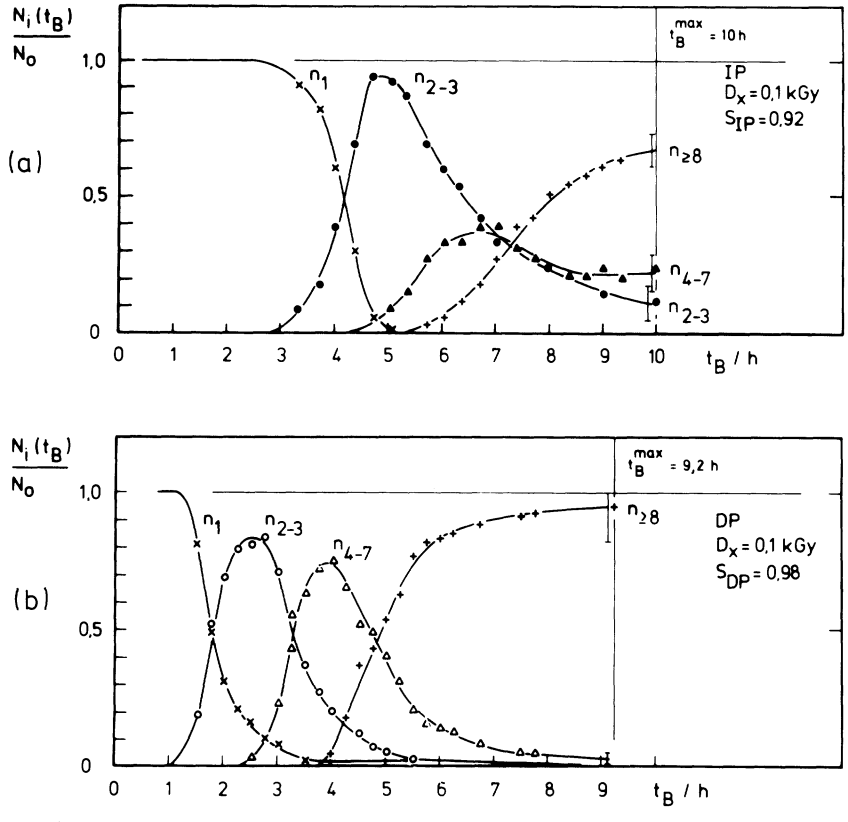

FIG. 6. Time-dependent behavior of relative cell groups $n_i(t_B) = N_i(t_B)/N_0$ of irradiated yeast cells (X-ray dose $D_x = 0.1$ kGy, early G_1 phase) growing for the time t_B on nutrient agar. [N_i is the fraction of single cells N_0 having produced at least $(i - 1)$ progeny.] (a) Immediate plating on the microscope slide after irradiation (IP). (b) Delayed plating after 48 hr liquid-holding repair (DP).

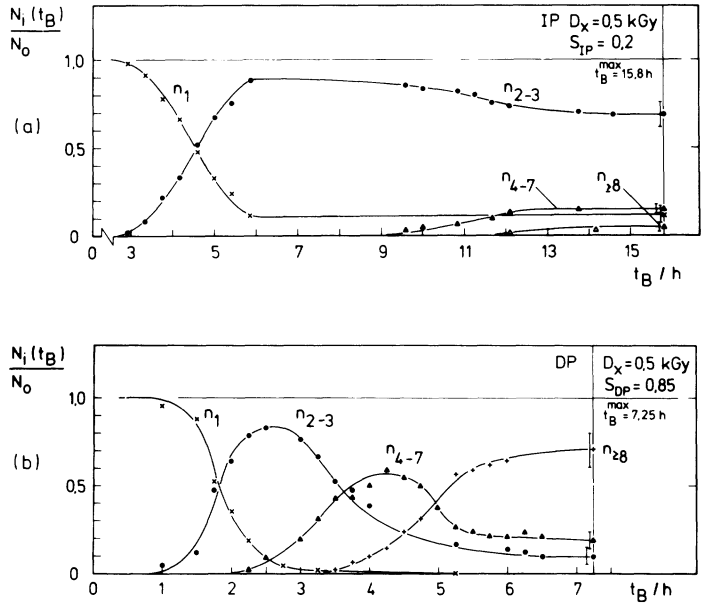

FIG. 7. Time-dependent behavior of relative cell groups $n_i(t_B) = N_i(t_B)/N_0$ of irradiated yeast cells (X-ray dose D_x = 0.5 kGy; early G_1 phase) growing on nutrient agar for the time t_B. [N_i is the fraction of single cells N_0 having produced at least $(i - 1)$ progeny.] (a) Immediate plating on the microscope slide after irradiation (IP) (b) Delayed plating after 48 hr liquid-holding repair (DP).

disturbed decrease of the n_{2-3} groups due to a delayed second division is correlated with a slow increase of the n_{4-7} group as can be seen very clearly in Fig. 7a. The n_{2-3} curve shows a near saturation indicating a defective second division (transition from the double to the four-cell colony). At a low dose (Fig. 6a) the n_{2-3} curve is decreased during the registration period ($t_B \approx$ 10 hr), whereas the n_{4-7} curve levels off, indicating that the number of cells leaving the n_{4-7} group to join the $n_{\geq 8}$ group is compensated by the cells joining the n_{4-7} group from the n_{2-3} group. The $n_{\geq 8}$ group represents the cells that survive radiation damage, as can be concluded from comparison with the colony tests. A later analysis will show whether these are the only colony formers.

After irradiation cells are observed at the end of observation that are not in the $n_{\geq 8}$ group. These belong partly to the fraction of inactivated cells (non-colony formers).

The distribution of the relative cell groups is drastically changed when the cells are allowed to repair radiation damage in nongrowth conditions (LHR) for 48 hr. At a low dose (Fig. 6b) the distribution of the relative cell groups at the end of observation [$n_{\geq 8}(t_B^{max}) \approx 0.95$] and the kinetics of the cell groups $n_i(t_B)$ are nearly the same as those of controls. The lower maxima of the n_{2-3} curves and n_{4-7} curves, however (Figs. 6b, 7b), and the remaining unsymmetrical curve shapes indicate that some division delays remain after repair. The reduction of the lag time (1.8 hr at D_x = 0.1 kGy; Fig. 6b) to a shorter value than that observed for controls is a char-

acteristic of the cell population used (early G_1-phase cells). This effect will be discussed in a later publication.

The following conclusions can be drawn from these qualitative results:

—The radiation effects on a cell population can be described with adequate sensitivity (*19*) by the time-dependent course of the relative cell groups (population spectrum) [see also (*19*)] derived from microscopic observations of a selected sample of single cells.

—The population spectra are remarkably influenced by different absorbed doses and by different repair conditions as can be seen from both the changed cell kinetics and the changed stationary distributions of the relative cell groups at the end of observation.

The descendants of unirradiated cells show the same morphology as the zero-generation cells. Irradiated cells, however, are heterogeneous and show abnormal shapes characterized by a large volume and a changed morphology during the time course of observation. About 50% of the irradiated cells and their progeny show these changes even at low radiation doses (about 0.1 kGy). The different cell shapes are distributed to the relative cell groups $n_i(t_B^{max})$ in the following way:

The irradiated single cells that remain unchanged, lyse, or shrink (about 10% of the irradiated single cells at doses up to 1 kGy) are assigned to the $n_1(t_B)$ group. Cells that obviously produce new cell nuclei without subsequent cell division and look like elongated ellipsoids are assigned to the cell group to which the number of generated new nuclei corresponds. The total number of irradiated cells showing this behavior was less than 6% in a sample of about 1000 cells. Even if such an abnormal cell is lysed later on it is assigned to the same cell group as if it were unlysed. Using these correlations all irradiated cells can be classified in special cell groups and the population can be described by the above population spectra.

To prove the validity of the method for detecting all the important reactions of irradiated cells within the possible intervals of observation (for instance, 16 to 20 hr) it is necessary to construct the complete time-dependent increase of the cell number of all observed pedigrees. With this integral description of cell growth the cell number can be seen to become stationary (Fig. 8) with increasing irradiation dose. The normalized integral increase $\Delta N(t_B)/N_0$ of irradiated single cells is shown with respect to time on nutrient agar after irradiation with different X-ray doses. The observed time-dependent increase corresponds to the fraction of surviving cells (surviving fraction in brackets). If the portion of inactivated cells is near 0.99 then no further growth is registered after 8 hr (within the margin or error of about ±5%). By this time the total population has nearly doubled in cell number even at high doses. An end of growth of the inactivated cells within these intervals has also been observed by other authors. The observation of inactivated haploid cells of the strain (211,1a) (*20*) shows a stationary cell group distribution after $t_B^{max} = 24$ hr. Irradiated single cells (n_1 group) and double cells (n_2 group) have been shown not to proliferate further even within 5 days after irradiation (*3*) and cannot increase their number of descendants. In some cases lysis occurs.

Hence it can be concluded that microcolonies of inactivated yeast cells are sta-

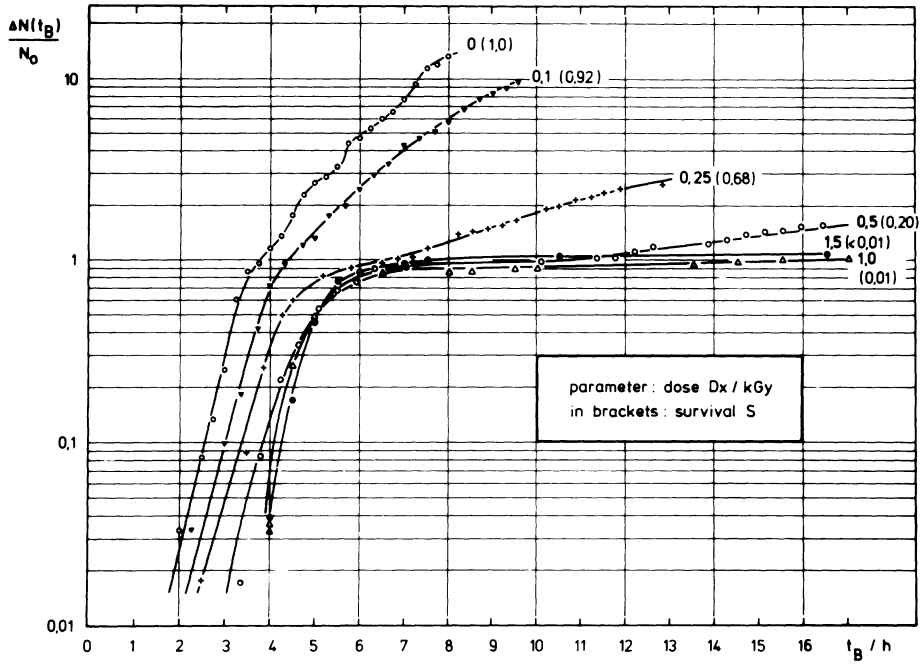

FIG. 8. Relative integral increase in cell number of a population of N_0 single cells in dependence on time t_B on nutrient agar (without repair; parameter: X-ray dose D_x).

tionary after an interval of about 20 hr. Therefore within the experimentally observed cell groups $n_i(t_B^{max})$ for $i \leqslant 8$ there must be a number $i = i_s$ (s = survivors) for which the following is true: when $i < i_s$ then

$n_{i<i_s}(t_B^{max})$ are the relative cell groups describing the inactivated zero-generation cells,

and when $i \geqslant i_s$ then

$n_{i\geqslant i_s}(t_B^{max})$ are then cell groups whose descendants are able to divide and eventually form macrocolonies of 10^5 to 10^6 cells.

The number i_s should now be defined in comparison with the survival curves of the cfa test. For this reason an analytical expression has to be defined which describes the ability of the cells to perform the division $j + 1$ subsequent to the division j.

The portion $N_i(t_B^{max})$ of the zero-generation cells N_0 has produced colonies with i cells after the time t_B^{max}. This cell number is correlated with the number of generations in which a single cell has generated descendants. For instance, $i = 1$ means that a cell did not divide and belongs to the zero generation, $i = 2$ means that the cell has produced a daughter cell within t_B^{max} and reaches the first generation although the mother cell is inactivated and so on.

The probability $p(0 \rightarrow j)$ that after irradiation a zero-generation cell reaches the generation $j (j \leqslant 3)$ is

$$j = 1\text{: } p(0 \rightarrow 1) = \frac{\text{cells, not remaining in the } N_1 \text{ group}}{\text{irradiated cells}}$$

$$= \frac{N_0 - N_1}{N_0} = (1 - n_1(t_B^{max})), \quad (2)$$

$$j = 2\text{: } p(0 \rightarrow 2) = \frac{N_0 - \sum_{i=1}^{3} N_i(t_B^{max})}{N_0} = (1 - \sum_{i=1}^{3} n_i(t_B^{max})), \quad (3)$$

$$j = 3\text{: } p(0 \rightarrow 3) = \frac{N_0 - \sum_{i=1}^{4} N_i(t_B^{max})}{N_0} = (1 - \sum_{i=1}^{4} n_i(t_B^{max})). \quad (4)$$

The division probability $p(0 \rightarrow j)$ describes the relative fraction of the irradiated cells that can perform at least j divisions. This is the fraction of the surviving cells correlated to the corresponding division j. The righthand side of Eq. (4), for example, is equal to the relative cell group $n_{\geqslant 5}(t_B^{max})$ that is able to reach the third generation characterized by a microcolony of five or more cells.

The division probabilities $p(0 \rightarrow j)$, $j = 1, 2, 3$, are shown half logarithmically in Fig. 9 versus different X-ray doses for immediately plated cells and for cells after liquid-holding recovery. The dashed lines represent the colony test survival curves. To maintain clarity in the plot in Fig. 9 only the error bars of the $p(0 \rightarrow 3)$ curves are drawn. These give the margin of errors of the sum of the mean $n_i(t_B^{max})$ values [see Eq. (4) and Fig. 6].

The dose–effect curves of the surviving cells give no detailed information about the inactivated cells but represent the sum of all possible cell fates characterized by the area above the survival curves. The division probabilities $p(0 \rightarrow j)$, however,

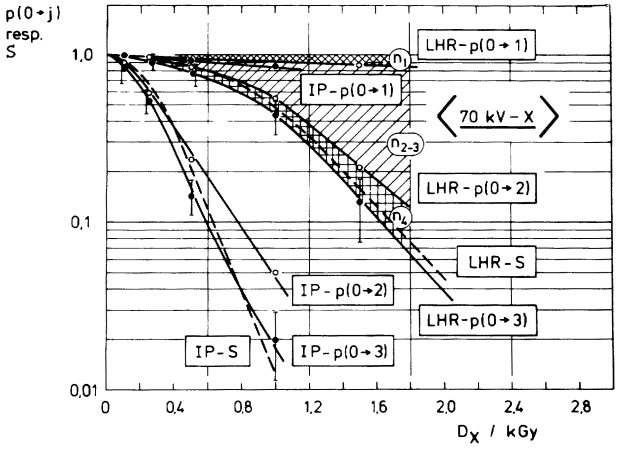

FIG. 9. Division probability $p(0 \rightarrow j)$ ($j = 1 \cdots 3$) of irradiated yeast cells (strain 211; early G_1 phase) in dependence on the X-ray dose D_x with (LHR) and without (IP) repair. (The corresponding survival curves S are dashed lines.)

allow an insight into the distribution of the fates of the inactivated cells. The area above the LHR-$p(0 \rightarrow 1)$ curve corresponds to the cells remaining as single cells [see Eq. (2)], the area between the LHR-$p(0 \rightarrow 1)$ and LHR-$p(0 \rightarrow 2)$ curves represent the final cell fates of the double and triple groups as the main contribution to the inactivated cells, and the cells able to form a four-cell colony are characterized by the area between the LHR-$p(0 \rightarrow 2)$ and LHR-$p(0 \rightarrow 3)$ curves. Within the margin of error, only the division probability $p(0 \rightarrow 3)$ corresponds with the cfa-survival curves for the IP and the LHR experiments (this fact will be confirmed for all used LET values later). Therefore, the survival of yeast cells can be defined as the probability of an irradiated cell producing a colony of at least five cells in the third generation. It is not necessary that all progeny cells divide regularly and produce cells to complete the pedigree of the third generation. The probability of producing only one cell in the third generation corresponds to the cfa-cell survival [see Eq. (4)].

Cells that have stopped their proliferation at least in the second generation can be selected by looking at the sum of pedigrees used for the definition of the relative integral cell number produced from cells irradiated with different doses (Fig. 8), and applying the above definition of cell inactivation. The relative integral cell number of these inactivated cells ($\Delta N_{(1-S)}/N_0$) can be constructed with respect to time and is shown in Fig. 10. After 12 hr the production of new cells is finished and the curves become stationary within an error of about ±5%, even after irradiation by low doses. This both confirms the claim that the observation time is sufficient for identifying inactivated cells and shows that no colony-forming cells are included in these cell groups causing the time-dependent increase in total cell number of Fig. 10.

CONCLUSION

In this report an automatic method is described for the observation of single cells within a proliferation period of four generations. The descendants of a single cell were microscopically registered up to a microcolony of eight cells, and these cells were sorted to give a pedigree. Furthermore all intervals were determined that are necessary for the analysis of the kinetics of the population.

By using a scanning microscope many cells can be automatically registered, thus guaranteeing a statistically secured interpretation. Because of the simple representation of the cells by dark rings on a light background this optical information can easily be read by a TV camera and the digital data obtained stored in a computer. Programs developed for pattern recognition methods and for the correlation of newly produced cells deliver the necessary data for differential analysis of cell inactivation.

This method allows the investigation of the time-dependent behavior of irradiated cells and a comparison with unirradiated cells, because the same growth rates are observed in the microscope chamber and in suspension. A limitation of this method could be that observation is limited to four generations. For yeast cells, however, it can be shown that all changes that are important for the phenomenon of cell inactivation occur within this interval; therefore the observation range is sufficient. Comparison with survival curves from tests of colony-forming ability of irradiated cells allows the correlation of the observed relative cell groups with these surviving cells. The distribution of cell groups, which do not divide and become finally stationary, can be correlated to division probabilities, indicating the possibility that a cell or its

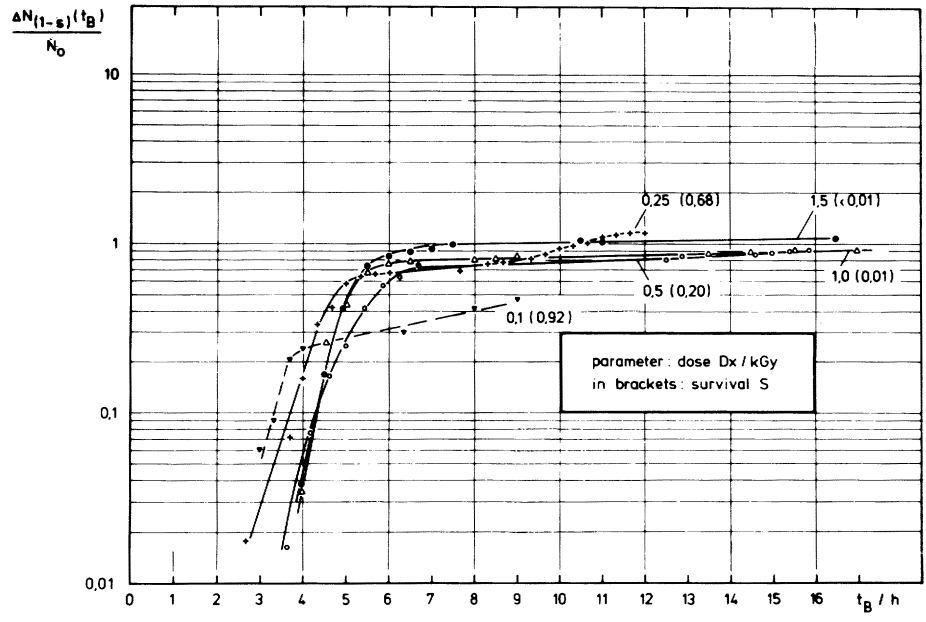

FIG. 10. Relative integral increase of the progeny of at least inactivated cells out of a population of N_0 single cells in dependence on time t_B on nutrient agar (without repair; parameter: X-ray dose D_x).

descendant will perform the first three postirradiation divisions. The division probability of a single cell of the generation zero for reaching the third generation equals the survival curve. This determines a single cell that produces a microcolony of at least five cells as a survivor able to form a macrocolony. Cells or their descendants, which remain in generations zero, one, or two, are declared to be inactivated.

The method allows time-dependent observation of an irradiated population and the recognition of inactivated cells. It will be used to investigate the effects of different ionization densities on yeast.

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