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DISCLAIMER

THE ACTION OF PENETRATING RADIATIONS ON YEAST CELLS*

by

Cornelius A. Tobias, Robert K. Mortimer, Ralph L. Gunther and Graeme P. Welch

Ever since Nadson and his associates (1) (2) demonstrated that x-rays affect cell division in yeast cells more than metabolism and that radiations cause morphological mutations in these cells, research with yeast cells has played an important role in understanding the foundations of radiobiology and radiation genetics. Holweck and Lacassagne (3) studied the dose effect relationship for survival and Latarjet and Ephrussi (4) brought the survival for the first time in relation to the ploidy of the cells. We wish to report here briefly on studies carried out in Berkeley on radiation-induced inhibition and delay in cell division, the production of mutations and the survival of cell populations exposed to x-rays continuously for many generations.

Life Cycle

Saccharomyces cerevisiae are found in nature normally in diploid vegetative clones, with their oval shaped cells about 7 x 6 microns in size. On nutrient medium of proper composition the cells may undergo budding and mitotic division at regular time intervals (5). Under certain nutritional conditions the diploid cells undergo meiosis to produce an ascus containing four haploid spores. The spores belong to opposing mating types and will conjugate if nutrient is supplied. Winge (6) developed a microdissection technique to isolate each of the four single haploid spores, which when separated may be made to grow in haploid, mitotically budding vegetative clones. By bringing haploid cells of opposing mating type into contact, diploid zygotes are formed which give rise to a hybrid diploid clone of cells. This cycle makes it feasible to do genetic analysis ("tetrad analysis") with unusual facility. The use of this method and the identification of many mutants with definite nutritional requirements, has led to detailed knowledge of the nature of inheritance in this organism. There is evidence for at least 8 linkage groups; therefore, the existence of up

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to 16 chromosome arms may be assumed. Unfortunately, these as yet have not been seen in the microscope. In the last few years more than 2,000 asci were dissected and analyzed by Hawthorne and Mortimer (7). It is well known since work of Ephrussi (8) that cytoplasmic inheritance also plays an important role in yeast genetics.

Occasionally, haploid cells will diploidize (9) by forming homozygous "illegitimate" diploids of the same mating type. These can be made to form zygotes with haploid and diploid cells of the opposite mating type, giving rise to zygotes which develop into clones of stable triploid and tetraploid cells. Extension of these techniques led Mortimer (10) to produce clones of pentaploid and hexaploid cells. Figure 1 shows some of the available clones of cells, all derived from the same diploid. Not only the content of genetic material, but size, DNA and RNA content tend to correlate with ploidy. Figure 1 is reminiscent of the beginning of an isotope table.

Radiation resistance and ploidy

Knowing of the profound effect of radiation on the nucleus, it seemed to be of interest to find out how radiation induced inhibition of cell division depends on the number of sets of homologous chromosomes in the yeast cell. Early work by Latarjet and Ephrussi (4), Beam (11) and Zirkle and Tobias (12) clearly showed that diploid cells are much more resistant to x-rays than haploid: when formation of microcolonies was studied, the ratio of 50% LD between diploid and haploid was about 5. At the present time, careful studies on macrocolony formation are available on clones of cells of all ploidies between one and six (10). In Figure 2, survival curves are presented for 50 kv x-rays for some closely related strains of various ploidies. The haploid cells are most sensitive and the diploids most resistant. Beyond diploid, the radiosensitivity increases with increasing genome number.*

Since the cytoplasmic contents of different ploidy cells appears to be similar, it was assumed that nuclear and perhaps genetic damage would have to account for the cell division inhibition. Analogy was made to the well-known genetics of drosophila, and experimental evidence was obtained for definite types of damage and repair mechanisms.

Recessive lethal damage (Type 1)

The first experiments suggested that the shape of haploid and diploid microcolony survival curves might be accounted for by a lethal chromosome damage which expresses itself in the haploid, but if it occurs in one genome only, it is recessive in the diploid. This model predicts

*Previously, unusually high radioresistance of a yeast believed to be polyploid was reported (13). On subsequent analysis attempts to sporulate were unsuccessful and the high ploidy could not be proven. This cell apparently belonged to the torulopsis strain.

that recessive lethals of this kind will increase the radiosensitivity of the diploid cell, in accordance with the proposal by Latarjet and Ephrussi (4) that x-rays may partially haploidize a diploid cell, and such change was indeed observed (14), although it has not been proven that it is due to recessive lethals.

A simplified mathematical model for survival has been proposed on the assumption that the chromosomes contain a finite number of "sites", which, either due to their biological importance or due to their physicochemical constitution, become focal points of genetic injury to cells. "n" such sites per genome are assumed. If the lethal damage is completely recessive and no recovery is possible, then the probability P_1 of a cell of ploidy p surviving a flux of F ionizing particles /cm^2 may be expressed as

$$P_1 = \left[1-(1-e^{-\sigma F})^p\right]^n \qquad (1)$$

where σ is the apparent "cross section" of a single sensitive site and it is assumed that all n sites have about the same cross section. The dose D in energy units and the particle flux F are related: $D = \epsilon F$, where ϵ is the linear energy transfer.

Wood (15) obtained $n \approx 30$ and n appears to be the same in the presence or absence of oxygen although in oxygen all cells are more sensitive to x-rays by about a factor of two. These determinations of n are not significant since subsequent work showed that recessive defects can not completely account for inhibition of cell division at higher ploidies. Radiation sensitivity varies with physiological state of the cell, and in the budding state, cells are about 10 times more resistant than resting (16).

Recessive lethals have been demonstrated in x-irradiated diploid yeast by sporulating the cells immediately following exposure to radiation and observing the ability of the haploid spores to form colonies (17). The procedure is shown in Fig. 3. Many of the haploid spores are not viable at a dose which inhibits cell division in only a few percent of the diploids while most diploid cells grow into colonies. The number of recessive lethals found by Magni (18) is much less, however, than that expected if all haploid cells are inactivated by this damage. Exact quantitative information on the percentage of recessive defect is difficult to obtain because some cells do not sporulate and cell division may bring about chromosome rearrangements.

Dominant lethal damage (Type 2)

It was reasoned from the nature of survival curves of higher ploidy cells that single ionizing events can give rise to lethal results even in the presence of multiple genomes. In some cases this finding may be a result of recessive injury by the same particle in a pair of allelic sites (19) but this mechanism accounts for only part of the effect with heavily ionizing α (alpha) rays. For x-rays the most likely explanation is the existence of dominant lethals induced by the radiation. Mortimer (20) exposed individual haploid cells to various doses of x-rays and subsequently

using micromanipulation techniques, brought these cells in contact with unirradiated haploid cells of the opposite mating type, as shown on Figure 4. Many of the zygotes formed failed to germinate and the zygote survival curve appeared to be similar to the survival curve of the same ploidy vegetative cells. The frequency of lethals increases at a given dose proportional to the ploidy (21). Thus in the presence of dominant lethal damage an expression for the probability P_2 of a cell of ploidy p surviving a flux of particles F could be

$$P_2 = f\ (p.F)$$

where P_2 is the dominant lethality function, similar to that defined by Lea and Catcheside (35). The dominant lethality function as obtained for haploid and diploid cells is shown on Figure 2.

If dominant and recessive lethal damage are independent and also are the only important mechanisms of cellular inactivation in yeast, then the overall survival probability would be

$$P = P_1 \cdot P_2 = \left[1 - (1-e^{-\sigma.F})^p\right]^n \cdot f\ (p.F)$$

For ploidies of triploid and higher

$$P = P_1 \cdot P_2 \quad f(p.f)$$

Thus the LD50 of cells decreases in an inverse fashion relative to ploidy, and this is approximately what is found.

Many diploid cells, survivors of a moderate dose of radiation, give rise on nutrient agar to microcolonies which proliferate at reduced rate and have greater radiosensitivity than normal diploids (14). Slow growing colonies are also produced as progeny of zygotes which originate from conjugation of one normal haploid and one irradiated haploid cell. These cells may be partially "haploidized" (4). Assuming that r sites are affected (12) (14), one would be left with a cell with r haploid and n-r diploid genetic sites. Probability of survival of the injured diploid (p=2) cells:

$$P = P_1 \cdot P_2 \cdot P_3 = \left[1-(1-e^{-\sigma F})^2\right]^{n-r} \cdot f(2F).e^{-\sigma rF} \qquad (3)$$

A large fraction of the progeny survivors of a moderate dose of x-rays exhibit increased radiosensitivity corresponding to eq (3). The number observed, however, is still definitely smaller than what may be calculated from probability distributions.

Actually, different strains of "normal" cells of the same ploidy exhibit definite variation in their radiosensitivity, related to their genetic constitution. The differences are much more marked in diploid than in haploid cells. Generally speaking, greater heterozygosity of diploid cells leads to increased resistance to x-rays. This is shown by the finding

that homozygous "illegitimate" diploids are more radiation sensitive than diploids obtained from mating.

Physico-chemical mechanisms

Interpretation of the physicochemical mechanisms of radiation injury is made more difficult by the fact that the initial injury is very dependent on the spatial distribution of ionization, on the water content and chemical composition of the medium, and on the physiological state of the cells. Recovery from part of the injury is also influenced by the same factors. The variation of the cross section σ was measured over a wide range of linear energy transfer (LET) (12) (22) (38) and the relative biological effectiveness (RBE) is found to rise over a considerable LET interval.* No one has as yet found chemical agents that would modify radiation damage at high LET (23), but oxygen and other modifiers will act on yeast cells at low LET. High temperature applied pre or post irradiation, amplifies radiation effect (24). Thus σ shows considerable variations. One can only explain the variations of σ by assuming that the radiation effect at low LET is qualitatively different from that at high LET. The meaning of σ is not the true cross section of some actual body of molecules where the ionizing particle must hit; it is rather a number involving probability of interaction of the ionizing particle with the nucleoprotein and surrounding cellular medium. However, ionization within essential macromolecular structures is frequently effective. Considerable evidence is accumulating that the site of the actual expressed injury may be removed from the actual site of ionizati n. One explanation given makes the assumption that most of the primary interaction need not take place in the macromolecule affected, but chemical intermediates may migrate to the site of injury and react there (12) (36) (37) (38). Another model assumes generation of injury within the affected macromolecule, which then chemically interacts with the surrounding cell medium (25). The effectiveness of high LET may then be ascribed to the production of several simultaneous ion pairs at or near the site of injury. Figure 3 gives graphic representation of the two models.

Post irradiation lesions

Submicroscopic radiation injury may recover or remain latent. For example, post irradiation temperature changes affect the ability of cells to form colonies (24). The radiolesions frequently come to expression during cell division. By observing the time sequence required for cell division and the fate of the progeny of irradiated cells under the microscope, it was ascertained that yeast cells show great statistical variation in their ability to produce viable daughters. A graph showing the time sequence of subsequent cell divisions in normal and in irradiated diploid cells is shown

*The latest RBE data obtained for haploid yeast cells are plotted in Figure 2 of "Biological and Medical Studies with High Energy Particle Accelerators" by J. L. Born et al. submitted to the 1958 International Conference on the Peaceful Uses of Atomic Energy.

on Figure 5. The normal cells show a regular pattern of cell divisons and nearly all cells divide. The progeny of irradiated cells not only show great variation in cell division time, but include production of many cells which are not viable (26). Abnormal generation of non-viable cells may go on for several generations. Several cells among the progeny require prolonged time for subsequent divisions which may persist for many generations. A similar effect on growth of macrocolonies was observed by Korogodin (39). When a chromosome rearrangement or mutation occurs that allows the cells to regain their normal ability to divide, the healthy cells will outgrow the ones with reduced viability (14), and this mechanism may, in the course of cell divisions, reduce the recessive lethals in the population.

Radiation induced mitotic segregation of genetic characteristics

Study of the alteration of phenotype and genotype of diploid Saccharomyces cerevisiae gave more definite evidence of the nature of genetic damage and its delayed phenotypic appearance. It is important to know at which level x-rays act on genetic constitution -- are single genes or their molecular configuration involved, are several genes affected together, or are intergenic linkages altered? So far it appears that point mutations, at a given locus, are rather infrequent. A surprising amount of chromosome rearrangement occurs, however, as a result of mitotic crossing over. James (27) has shown that diploid yeast, heterozygous for a locus determining ability to ferment galactose, will produce a relatively high frequency of aberrant colonies when irradiated with ultraviolet. Whereas, unirradiated cells all gave rise to black colonies on indicator agar, many irradiated cells produced colonies which were entirely white or sectored black and white. The white phenotype indicates homozygosity for the recessive locus conferring inability to ferment galactose. Genetic analysis of some of the sectored colonies showed that most were homozygous dominant on the black side and homozygous recessive on the white side. These results give strong support for the occurrence of mitotic crossing-over in u.v. irradiated yeast. These effects have also been shown to occur in x-irradiated diploid cells heterozygous at a number of biochemical loci. The frequency of occurrence of variant colonies was found to increase with the distance of the locus from its centromere.

Considering the results for the ad_2 locus, a dose of 10,000 r, which produces only 10% inactivation, gives rise to 3.5% variant colonies. These variants express changes on only an arm of one of the linkage groups. There is evidence for at least 8 linkage groups in yeast and hence up to 16 arms. Assuming a uniform population of cells, and a uniform sensitivity per chromosome arm, the minimum percentage of diploid cells with one or more genetic alterations which result in homozygosis is:

$$100 \left[1 - (1 - 0.035)^{16} \right] \approx 45\%$$

Thus, on the basis of the above assumptions, nearly half the cell population has experienced an alteration of genotype at this nearly sublethal dose.

REVERSIONS TO WILD-TYPE

Mortimer and Gunther (30) are carrying out a systematic study on factors affecting x-ray induced reversion mutations. Irradiation of the homozygous diploid auxotrophe (i.e., requiring nutritional metabolite) caused back mutations, viz, reversion to the prototrophs (not requiring some metabolite). The reversion rate in several different strains was proportional to dose (in the range where the lethal effect from the radiation remained small). Reversion rates induced varied from strain to strain in a fifty-fold range. The reversion rate increased in the presence of oxygen over that obtained anaerobically, but the nitrogen-oxygen dose reduction factor (DRF) showed considerable variations between 1.5 and 3 in agreement with similar observations of Anderson (31) on bacteria. Since inhibitinn of cell division has a DRF of about 2 to 2.2, this evidence contributes to the belief that detailed mechanisms of point mutation effects may be different from cell division inhibition.

It is now clear that attempts to make mathematical models for survival are complicated by the finding that frequent genetic crossovers result and the probability of crossover depends on locus involved. One very frequent result of irradiation of diploid cells is subsequent change from heterozygosity to homozygous nature. In the sublethal dose range many such changes appear detrimental. However, a nutral result of a small dose might be elimination of some existing recessive lethals from the population and, at the expense of producing some nonviable cells, the appearance of homozygosity of good, dominant characters. Roman and Jacob (28) have taken diploid yeast cells heteroallelic for a biochemical requirement locus, from which recovery by reversion mutation is possible. Such cells ordinarily do not form colonies on nutrients lacking the particular substrate involved. Small doses of ultraviolet rays significantly increased the reversion rate. In our laboratory (29) a similar experiment was tried using x-rays at a heteroallelic arginine requirement locus. Fifty roentgens doubled the reversion frequency.

YEAST POPULATIONS CONTINUOUSLY EXPOSED TO X-RAYS

It is of general interest to know how the knowledge gained in acute genetic and lethality experiments may be applied to populations of cells continuously exposed to x-rays for many generations, particularly in view of the fact that radiation sensitivity varies over a tenfold range during the budding cycle (16), that chromosome crossovers are quite numerous in irradiated populations, and the assumption that mutations will accumulate.

An actively dividing diploid yeast cell population in a liquid culture medium has been exposed by Welch to 85 kvp x-rays (32) for various periods up to 115 generations. The continuous flow apparatus which maintained the population in the steady state was suggested by previous experiments (33) (34). We incorporated modifications that permitted cell divisoon at a maximum rate, not limited by chemical environment. Steady states of population were brought about by varying the rate of nutrient flow to the growth tube until the rate of washing cells out was matched to the rate of cell

division. The division rate at any time was then simply proportional to the known nutrient flow rate to the growth tube.

By modifying the rate of nutrient flow, it was possible to maintain a steady state population actively dividing in log phase at all dose levels tested up to 6150 r per generation. The main effect on the population seemed to be a lengthening of the period of time required for cell division. Figure 6 shows the dependence of yeast cell division rate on x-ray dose rate obtained for 26 different steady-state conditions. The decrease in division rate with dose rate is nearly linear at 1×10^{-4} $hr^{-1}r^{-1}$ generation out to about 2500 r/generation. Then it tends to level off up to 6150 r/generation, the maximum available for these experiments. Except for data at low dose rates, these results are in agreement with the radiation induced division delay model proposed by Burns (5). This is based on the assumption of a specific number of sites concerned with delay and that delay is proportional to the fraction inactivated by radiation.

Several morphological changes of a general nature were noted in continuously irradiated populations. Without irradiation cells from the culture appeared quite uniform in size, but under the influence of x-radiation a spectrum of size was observed covering a range from normal up to 2x diameter. Moreover, with irradiation a marked increase in visible cytoplasmic structures of a granular nature was noted in the phase microscope. The attainment of steady-state under irradiation was marked by a transition period of several generations during which the average division rate decreased and the morphologic changes noted above took place. Likewise upon removal of radiation from the culture, several generations intervened before the original division rate was regained.

Tests for viability of the culture were made by plating samples from the growth tube and, after 24 hour incubation, counting viable and nonviable cells under a microscope. At 6150 r/generation 87% were viable, the dependence being approximately linear with dose rate.

During the longest run the accumulated dose to the ancestral populations was approximately 4×10^5r. Since exposure to sublethal x-rays enhances mitotic crossing over which in yeast is a major cause of genetic variation, the delivery of a large dose presents the possibility of selection of a variant better able to resist the damaging effects of radiation. On testing for this, no significant change in sensitivity to acute 50 kvp x-ray exposure was found in 6 samples taken during the course of the experiment which covered 115 generations.

CONCLUSION

Several features of the inhibition of cell division of yeast cells have been elucidated as a result of studies by a group of investigators over several years. The production by x-rays of recessive lethals and of dominant lethals has been shown, and these two seem to account for the most part for the inhibition of cell division by x-rays in various ploidy cells.

Genetic rearrangements in the diploid cell go on for several generations and result in lethals as well as in impaired and recovered cells. One mechanism involved in the rearrangements is x-ray induced crossover. The frequency of crossover is a function of linkage, and the induction of reversion mutations by x-rays shows great variations in rate of reversion as well as in oxygen sensitivity. Steady state populations of cells can exist under high dose rates of continuous irradiation.

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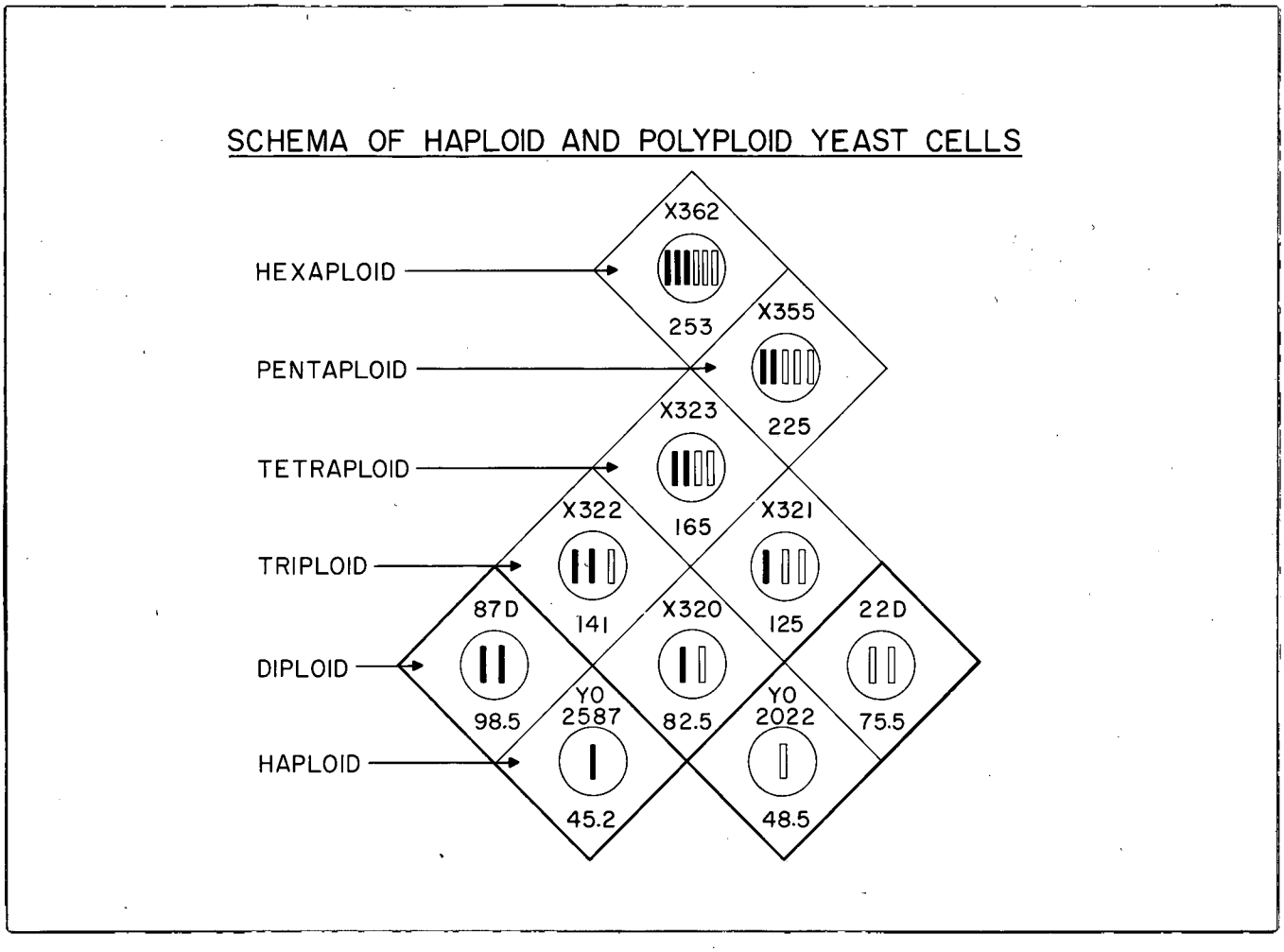

FIGURE 1

Schematic arrangement of some of the haploid and polyploid Saccharomyces cerevisiae used in Berkeley. Black and white bars refer to genomes of the two mating types. Cells on the left side of the axis will generally form zygotes with cells on the right side. The clone designation and cell volume in μ^3 are also given. Cells enclosed in the squares with thick borderline are the principal mating types. All other ploidies can be made to sporulate; on rare occasions they can be made to form zygotes with another mating type.

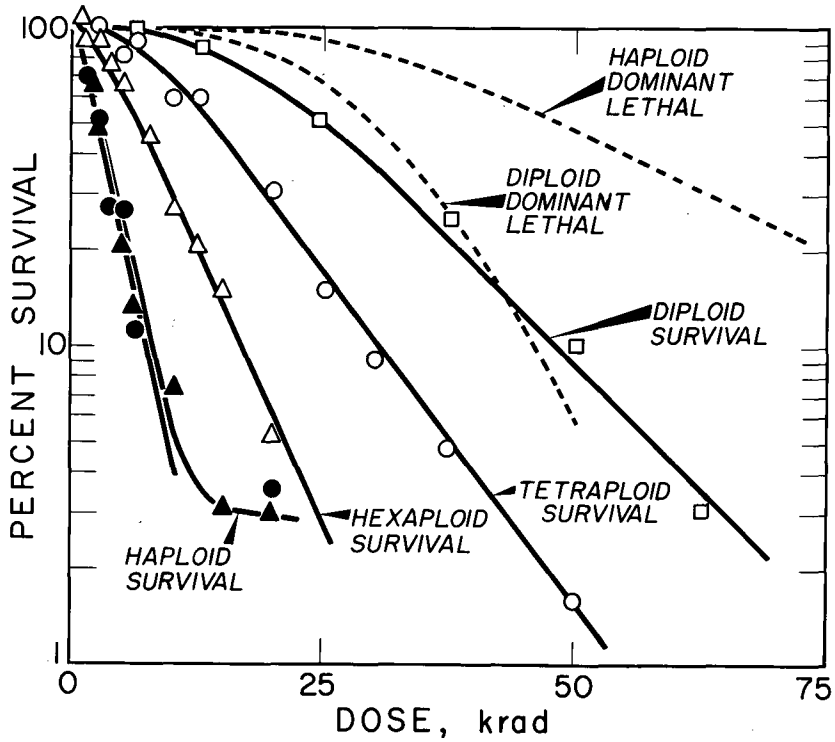

FIGURE 2

Survival curves of haploid, diploid, tetraploid and hexaploid Saccharomyces cerevisiae. Criterion for survival was formation of macrocolonies from single cell isolates. 50 kv x-rays were used at room temperature. Probability function P_2 for dominant lethals as described in text is also plotted.

●	haploid	Y02587
▲	haploid	Y02022
□	diploid	X32
○	tetraploid	X323
△	hexaploid	X362

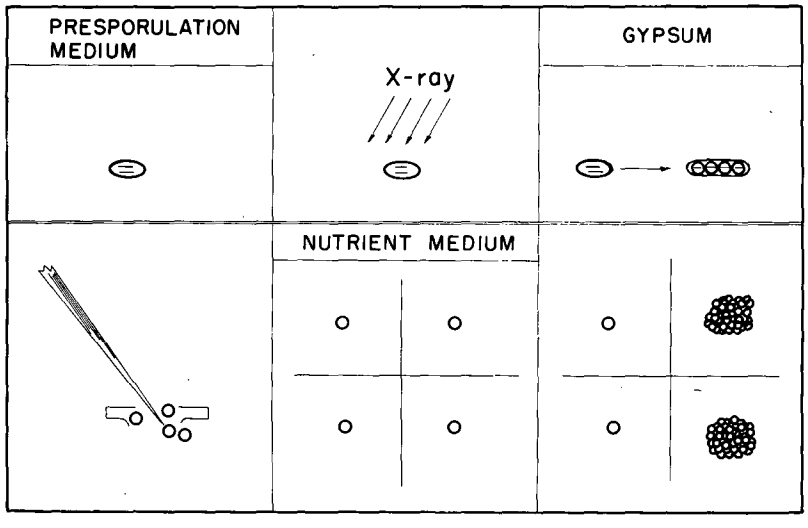

FIGURE 3

Procedure employed in sporulation. First diploid cells are grown in a rich, pre-sporulation medium. Following x-irradiation the cells are placed on gypsum slants, where after some elapsed time they sporulate. The ascospore is punctured with a microneedle, and the spores are separated and placed on nutrient medium. Clones should appear in 2 of 4 or 0 out of 4 spores from cells with recessive defects unless recovery occurs.

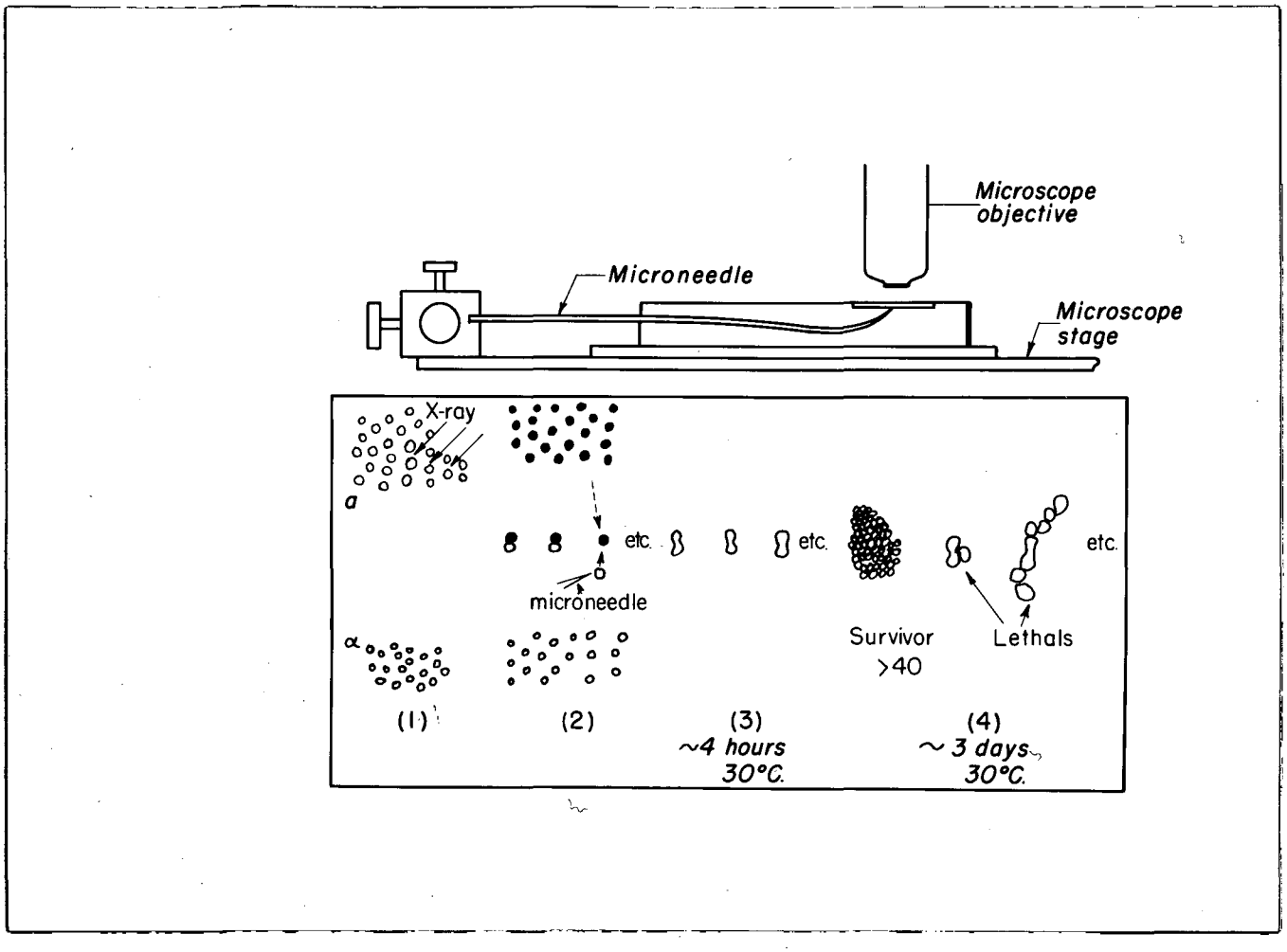

FIGURE 4

Procedure in studying dominant lethal damage in yeast cells.

Top: Arrangement of microneedle and microscope stage for manipulation of the cells. The cells are on the lower side of a thin layer of agar, mounted on a cover slip.

Center: (1) Cells of mating type a and α are placed on nutrient agar in two different locations. "a" cells are x-rayed, "α" cells shielded.

(2) By micromanipulation a and an α cell are brought into contact.

(3) In a few hours zygotes are formed.

(4) In a few days the zygotes give rise to clones (survivor) or to lethals.

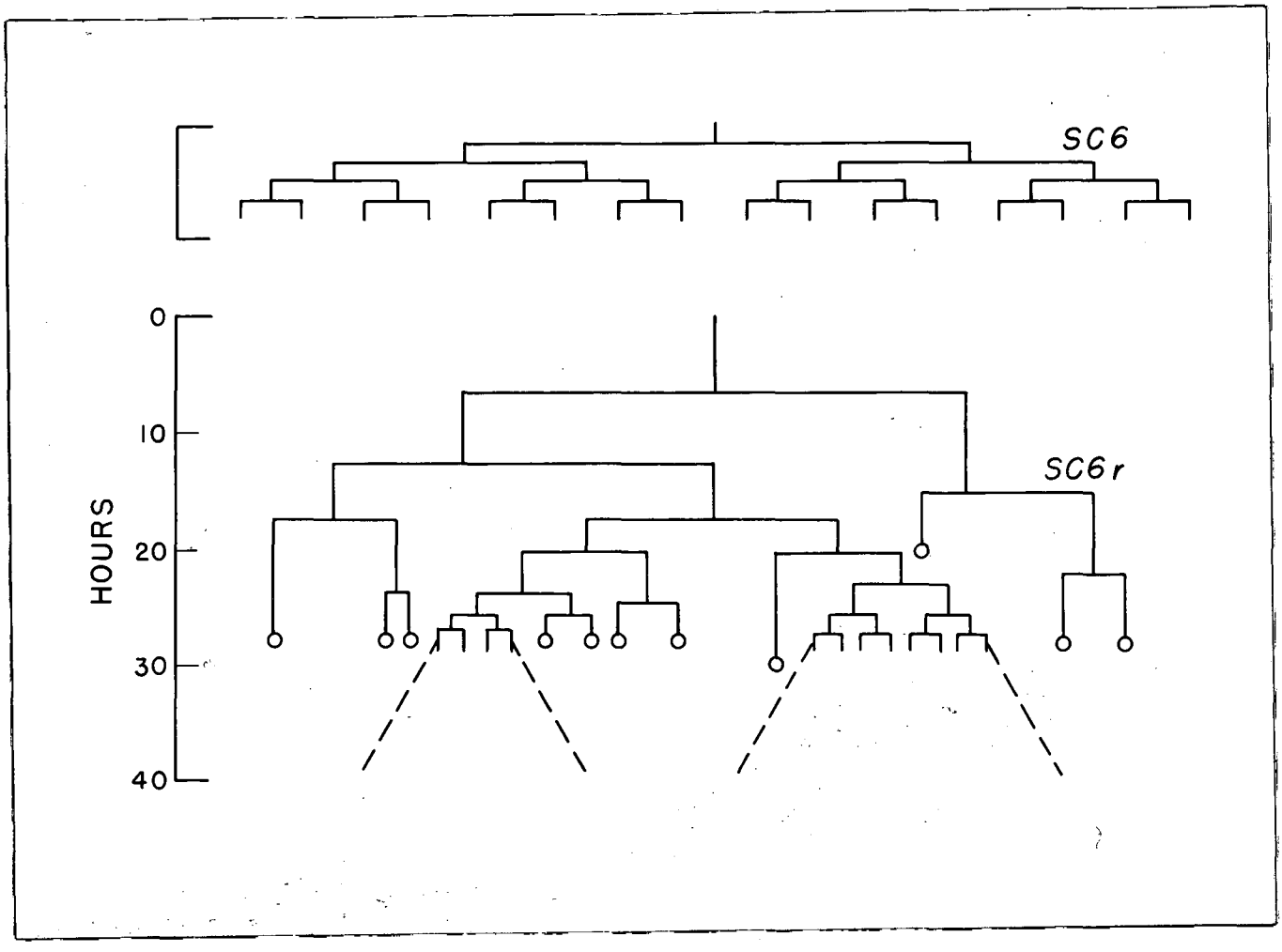

FIGURE 5

Budding times of normal diploid Saccharomyces cerevisiae (top) and of the progeny of a cell irradiated by 20,000 roentgens (bottom). Small circles indicate that the particular cell has not gone on to divide further. This study was carried out by micromanipulation techniques. Individual cells were observed under the microscope continuously, and mother and daughter cells separated by use of a microneedle after each division. Usually, yeast cells exist in the two celled state and separation of the two cells is possible only about the time when a new bud appears on one of them.

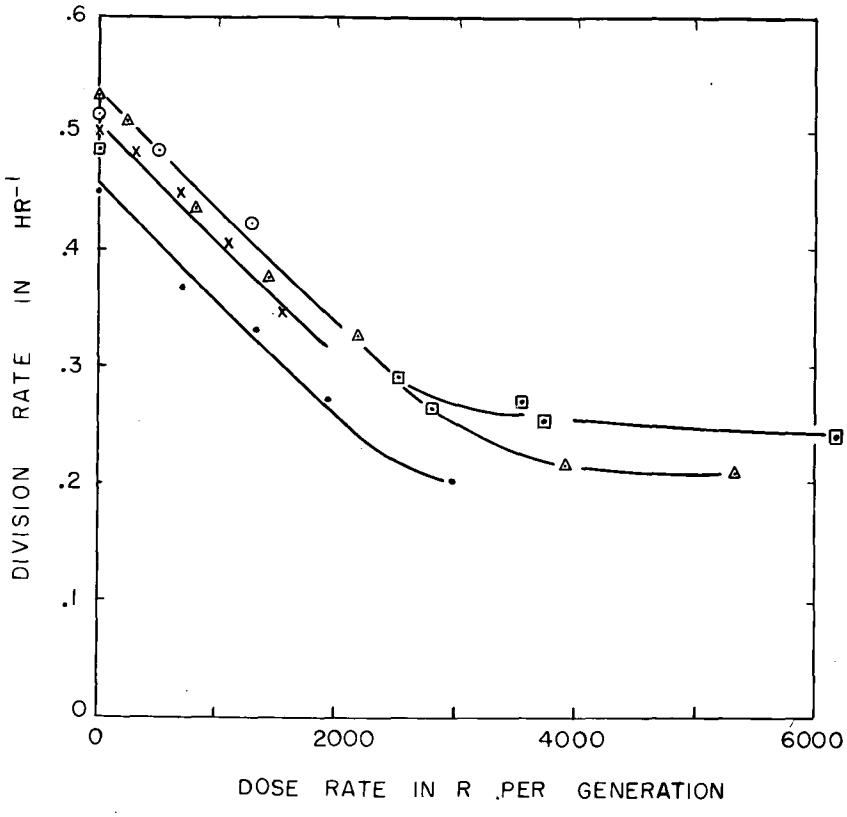

FIG. 14 DEPENDENCE OF DIVISION RATE ON DOSE RATE FOR DIPLOID *S CEREVISIAE* EXPOSED TO CHRONIC X-RAY IRRADIATION

FIGURE 6

Dependence of division rate on dose rate for diploid Saccharomyces cerevisiae exposed to chronic x-ray irradiation.

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