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DARK RECOVERY OF YEAST FOLLOWING IONIZING RADIATIONS

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Research and Development

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DARK RECOVERY OF YEAST FOLLOWING IONIZING RADIATIONS

John T. Lyman

(Ph. D. Thesis)

March 30, 1965

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ABSTRACT

Following exposure of diploid yeast to either x rays, ultraviolet light, or nitrogen mustard, a great increase in viability is observed if the irradiated cells are stored in distilled water (30°C) for 1 to 4 days before plating. Possible mechanisms which have been proposed to account for these results are (a) replacement of the damaged regions by de novo synthesis of DNA; (b) genetic exchange or recombination between homologous DNA molecules either during DNA replication or during chromosomal pairing; (c) direct enzymatic repair of damaged molecules.

Since there is a general idea that the biological effects of high-LET radiations are to a large extent irreversible, this paper is directed at determining the relationship between the recovery and the LET of the radiation, and will attempt to differentiate between the proposed recovery mechanisms.

The results of irradiation with x rays and heavy-ion beams have demonstrated that the recovery mechanism operates independently of the LET of the radiation, and also operates independently of the glycerol protection mechanism.

Experiments designed to lucidate the nature of the recovery mechanism have shown that besides the observed increase in viability, there is also a decrease in the induced allelic recombination frequency, a decrease in the time to form a visible colony, and a decrease in the dependence upon nutrient co-factors for the expression of the induced

allelic recombination when the irradiated cells are stored in a media lacking a nitrogen source before plating.

These results have been compared with the results expected on the basis of the different proposed recovery mechanisms. These comparisons suggest that the most likely recovery mechanism may be based upon the reunion of broken segments of DNA or upon enzymatic repair of damaged segments.

I. INTRODUCTION

Yeast cells have been used as test objects for studying the biological effects of ionizing radiations for many years. One of the earliest workers, Nadson (1, 2), demonstrated that x rays affect cell division more than the metabolism of yeast cells. This demonstration was followed by studies of the dose-effect relationship for cell survival by Holweck and Lacassagne (3, 4). After the establishment of yeast strains with known ploidies, R. Laterjet and B. Ephrussi (5) extended the survival curves to strains of ploidies other than the naturally occurring diploid strain. It was found that diploid and higher ploidies were characterized by sigmoid survival curves, while haploid yeast was inactivated exponentially. Other differences between haploid and higher ploidies have been observed (6-11). For instance, microscopic observations of irradiated yeast cells have shown that diploid cells almost always divide at least once before death ensues, while haploids are often stopped before a bud is produced (3, 5, 12, 13). Diploids, or their descendents, appear to exhibit a delayed recovery after irradiation, illustrated by the appearance of new clones 5 to 6 days after irradiation. The haploid cells, which survive at least two divisions, all have developed into colonies within 2 days (11, 14). Colonies formed by irradiated diploid yeast cells are much more heterogeneous in size than those formed from irradiated haploid cells (11, 14, 15). Division delay caused by ionizing radiations is much more pronounced in diploid than in haploid cells (8). The fact that in diploid cells there can be a delay in division which is not permanent and that eventually normal division can ensue suggests that there is recovery from some of the effects of ionizing radiations (16).

Exposure of Saccharomyces ellipsoideus and Bacillus paradysenteriae suspensions to low temperatures after x-ray irradiation considerably increases the number of surviving cells (17). It has been shown (18-20) that the introduction of certain antimetabolites

(3, 4-benzypyrene, sodium azide) into suspensions of irradiated microorganisms also increases the number of organisms that survive. Microorganisms have been reactivated by cultivation on suboptimum media (21, 22), or by temporarily storing the microorganisms in water (23). Recovery of diploid, but not haploid, yeast cells in water has been recently reported by Korogodin (24), and has since been elaborated on by Korogodin and others (25-39).

Patrick (37) has presented considerable evidence supporting the view that if yeast cells are inactivated by agents which are presumed to cause inactivation, primarily through damage to DNA, then under suitable conditions recovery mechanisms can enhance cellular viability. However, the magnitude of this recovery may vary widely, depending on the mode of inactivation. It has been suggested by Patrick that this variation may arise if: (a) the agent induces many types of molecular lesions, only some of which are amenable to repair; (b) all the damage is potentially repairable, but some may be so extensive it cannot be completely repaired by the existing recovery system or systems; (c) the agent is capable of interacting with and damaging the recovery mechanism itself. If densely ionizing radiations produce molecular lesions which were significantly different from those produced by lightly ionizing radiations, then one may expect a different degree of recovery following inactivation by these two types of radiations.

Inactivation of bacteria (40-42), yeast cells (14, 43–45), and mammalian cells in tissue culture (46-51) by fast, densely ionizing particles has resulted in a very similar relationship between relative biological effectiveness (RBE) and ionization density or stopping power (dE/dx). In all these cases, as the stopping power is increased the RBE increases until it reaches a maximum at dE/dx values around 2000 MeV-cm^2/g. Further increases in dE/dx result in decreases in RBE values.

Experiments by Powers (42) with Bacillus megaterium spores have demonstrated the lack of ability to modify their radiosensitivity at very high stopping powers ($dE/dx = 5500$ MeV-cm^2/g) by changes in water vapor pressure, by gaseous environment during irradiation, by postirradiation radical scavenging with H_2S gas, or by "deuterating" the spores by growing the bacteria in a medium containing D_2O substituted for H_2O. All these environmental conditions have been shown to be effective in modifying the colony-forming ability following irradiation with less densely ionizing radiations.

Elkind and Sutton (52) have shown the partial recovery of mammalian cells following x-ray inactivation. Further experiments by Todd (51), utilizing heavy-ion irradiation, have established that mammalian cells do not recover from irradiation by particles with dE/dx values greater than 2200 MeV-cm^2/g. Recovery of yeast has been demonstrated following α-particle inactivation (36).

The results of the aforementioned authors lead us to the conclusion that there is a system, or combination of systems, for cellular recovery that operates prior to the first division following irradiation. If the primary damage is to the DNA molecules, then they are being repaired. If some part of the damage is extra-DNA, then it is being nullified before it damages the DNA. Possible mechanisms to repair this damage might be based upon (a) replacement of the damaged regions by "de novo" synthesis of DNA; (b) genetic exchange or recombination between homologous DNA molecules either during DNA replication or during chromosonal pairing; (c) direct enzymatic repair of the damaged molecules.

Since there is a general idea that the biological effects of high linear-energy-transfer (LET) radiations are to a large extent irreversible, this paper is directed at determining the relationship between the recovery and the LET of the radiation, and attempts to differentiate between the different possibilities for a recovery mechanism by use of genetically marked yeast strains in which sporulation and recombination can be clearly distinguished.

II. METHOD AND MATERIALS

A. Biological Materials

1. Yeast

The following strains of the yeast, Saccharomyces cerevisiae, were used for these experiments.

X481, a diploid, obtained from Dr. R. K. Mortimer. This strain was developed as a hybrid between two related haploid strains, and has been used in other studies involving heavy-ion irradiations (45). The genotype of X481 is

$$\frac{a}{\alpha}\ \frac{hi_{5-2}}{hi_{5-2}}\ \frac{tr_1}{tr_1}\ \frac{AD_2}{ad_2}\ \frac{+ar_{4-1}\,AR_{4-2}}{p_1AR_{4-2}\,ar_{4-2}}\ \frac{thr_1}{THR_1}\ \frac{me_1}{ME_1}\ \frac{UR_1}{ur_1}\ \frac{ly_1}{LY_1}\ \frac{LE_1}{le_1}\ .$$

[The symbols a and α represent complementary mating type alleles. The symbols hi, tr, ad, ar, thr, me, ur, ly, le indicate ability (upper case) or inability (lower case) to grow in the absence of histidine, tryptophan, adenine, arginine, threonine, methionine, uracil, lysine, and leucine respectively. The subscripts identify particular genes and alleles.]

X841p, a petite mutant of X481. Respiratory-deficient yeast arises spontaneously from normal cells, and under most conditions constitutes approximately 1% of a growing population (53). The X841p was obtained from a YEPD plate containing unirradiated cells of X841. The strain was identified by its colony morphology, which was smooth, white, and smaller than the normal colonies. Its respiratory deficiency was tested by failure to grow on media in which glycerol was supplied as the carbon source.

XJ4, a homozygous mating-type (α α) diploid which had been obtained by mass-mating two α-mating-type haploid strains of differing genotypes. The homozygous diploid was then isolated by prototropic selection.

XJ4 has the genotype

$$\frac{\alpha}{\alpha}\ \frac{thr_4}{thr_4}\ \frac{tr_1}{tr_1}\ \frac{ad_1}{AD_1}\ \frac{ad_2}{AD_2}\ \frac{me_{10}}{ME_{10}}\ \frac{ur_1}{UR_1}\ \frac{LE_1}{le_1}\ \frac{AR_4}{ar_4}\ .$$

2. Media

YEPD. Complex medium for stock maintenance, routine culturing, and viability assay. Difco yeast extract, 1%; Difco Bacto-Peptone, 2%; dextrose, 2%; and agar, 2% (2% agar was omitted for liquid media).

YED. Complex medium used for some colony growth rate studies; YEPD with 2% Bacto-Peptone omitted.

PET. A medium containing glycerol as a carbon source, used to score respiratory deficiency (petites); glycerol, 3%; dextrose, 0.025%; Difco yeast extract, 1%; Difco Bacto-Peptone, 2%; and agar, 2%.

M-VIT. Minimal medium plus vitamins: Difco yeast nitrogen base without amino acids, 0.67%; dextrose, 2%; and agar, 2%.

SC. Synthetic complete. M-VIT supplemented with adenine, 20 mg/liter; lysine, 20 mg/liter; histidine, 10 mg/liter; methionine, 20 mg/liter; tryptophan, 20 mg/liter; threonine, 100 mg/liter (filter-sterilized and added to the media after media was autoclaved 15 minutes at 15 psi); and uracil, 20 mg/liter; leucine, 30 mg/liter; arginine, 20 mg/liter.

-X. Omission media, SC with one of the supplements omitted. These media are designated by the omitted nutrient, e.g., -ar means SC without arginine.

B. Radiation Sources and Dosimetry

1. x Ray

The x-ray source used was a Machlett OEG-60 beryllium-window tube. No additional filtration was used. The tube was operated at 50 kV and 25 mA, which resulted in a dose rate of approximately 15 krad/min at 15.4 cm from the target. Dosimetry of these x rays has been discussed by Mortimer (54) and by Birge, Sayeg, Beam, and Tobias (55). These authors concluded that the absorbed dose in rads to

a monolayer of cells is numerically equivalent to the air dose in roentgens, with a standard error or ± 10%, which is due to uncertainties in the composition of yeast and in the calculated absorption coefficients.

2. Heavy Ions

The heavy-ion source was the Lawrence Radiation Laboratory's heavy-ion linear accelerator (Hilac) (56). During the course of these experiments, the following heavy ions were used: deuterons, helium, lithium, boron, carbon, nitrogen, oxygen, fluorine, neon, silicon, sulfur, and argon. The Hilac accelerates these ions to a maximum energy of 10.4 ± 0.2 MeV/amu. The Hilac was operated at 12 to 15 pulses/sec with a pulse duration of 2.5 msec. Dose rates varied from 10 to 25 krad/min, depending upon the heavy ion used. The dose rate was adjusted in such a way that the irradiation time for a given degree of inactivation was approximately equal to the x-ray irradiation time for the same degree of inactivation.

3. Heavy-Ion Irradiation Equipment and Dosimetry

The experimental setup is diagrammed in Fig. 1. After the ions have received their final acceleration from the Hilac, the beam is focused with a quadrupole magnet, then deflected 12 deg from its original direction by a steering magnet to insure homogeneity of particle momenta. The beam then passes through the "monitor section," which is composed of a remotely controlled Faraday cup, and a combination scattering and beam-monitoring foil which is contained in an electrically insulated "Ozalid holder." The Ozalid holder allows foils to be removed and replaced without disturbing the main vacuum system. In order to facilitate alignment of the beam, it is sometimes useful to insert a piece of Ozalid paper into the Ozalid holder in place of the scattering foil. When the Ozalid paper is exposed to high doses of irradiation, the paper becomes charred where the ions have passed through it. This "burn pattern" can be useful in determining the beam's position within the beam pipe and in determining the sharpness of the focusing properties of the quadrupole magnets located within the drift tubes of the machine and also in the experimental area in front of

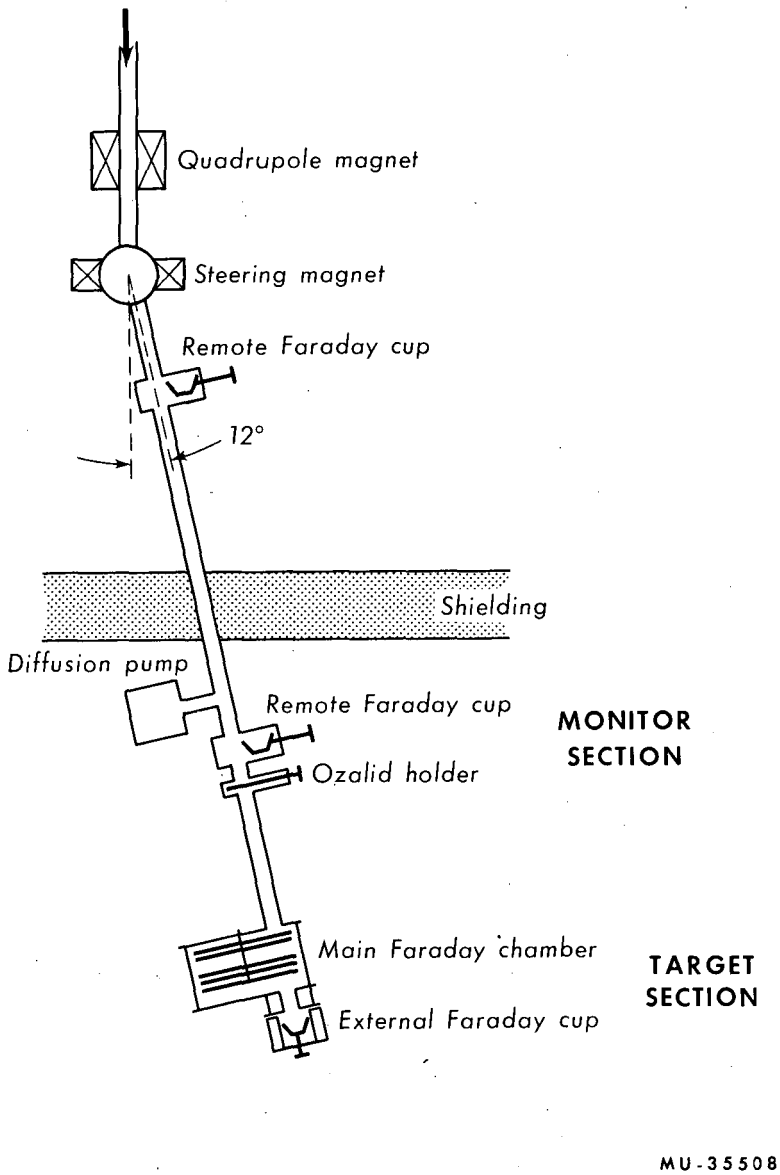

Fig. 1. Hilac irradiation experimental setup. The distance from the steering magnet to the target section is approximately 8 meters.

the steering magnet. The scattering foil is customarily 0.00025-in. Al, but can be easily changed if it is necessary to induce a greater angular divergence of the beam in order to obtain a more uniform distribution of particles at the position of the sample to be irradiated. The "target section" consists of an electrally insulated Faraday cup with a removable back, which facilitates the attachment of additional experimental equipment. This cup, which is called the "main Faraday chamber," is provided with an extension into the beam pipe, as shown in Fig. 2. The beam-defining aperture is just in front of the main Faraday chamber. The aperture and the Faraday extension are both within the field of a permanent magnet which surrounds the beam pipe. This magnetic field serves two purposes: (a) it prevents electrons which may be in the beam (ejected from the foil in the monitor section or ejected from the aperture) from entering the main Faraday chamber; (b) it prevents electrons ejected from the main Faraday chamber from scattering out of it.

The main Faraday chamber contains three absorber wheels, each with nine Al foils of measured thicknesses and one blank hole to allow the beam to pass through without energy loss. With this arrangement, a large number of different absorber thicknesses are available. The main Faraday chamber also contains two sample wheels for exposing biological samples, in a vacuum, to heavy ions. These sample wheels were not used during the investigation reported here. All five wheels can be rotated independently by means of remote control from a panel in the control area.

Attached to the main Faraday chamber, in Fig. 1, is the "external Faraday cup." This cup is insulated from the main Faraday chamber and is also guarded by a permanent magnet. The external Faraday cup may be replaced by a 0.001-in. Dural window which allows the beam to be brought out of the vacuum system, facilitating alignment of the apparatus, as well as aiding in the study of the homogeneity of the beam cross section.

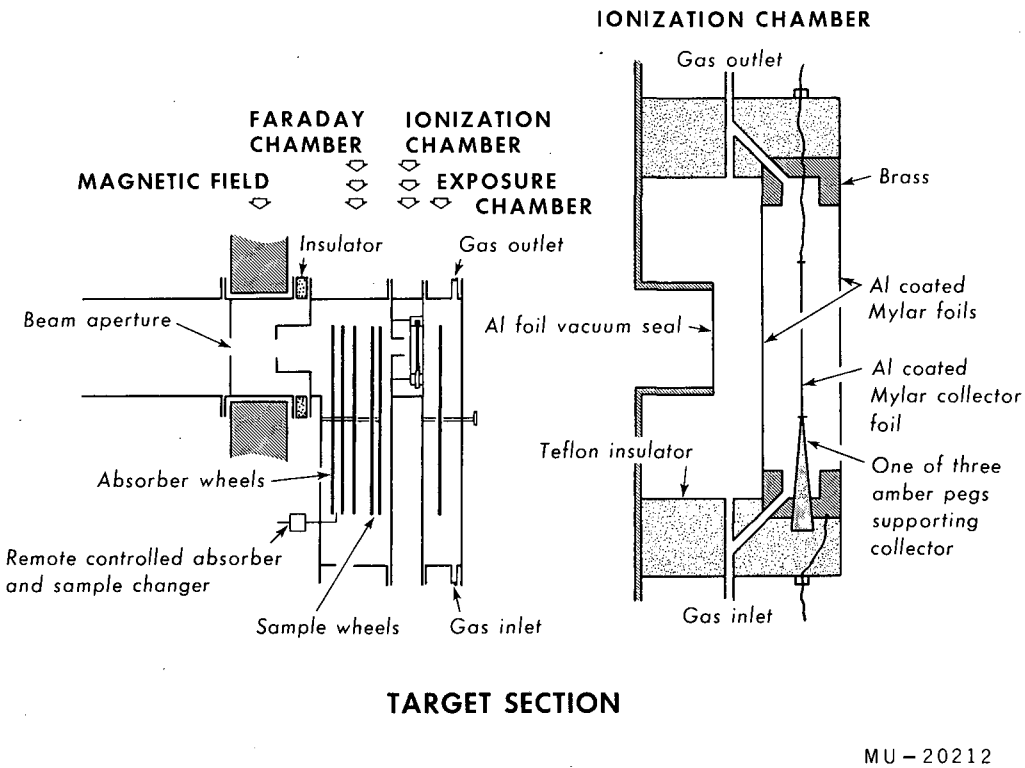

Fig. 2. The target section.

For exposure of biological samples in a wet state and in controlled gaseous atmospheres, an ionization chamber and an accompanying bombardment chamber were substituted for the back cover plate of the main Faraday chamber, Figs. 2 and 3. The design of the ionization chamber has been discussed by Brustad (57). The chambers currently being used differ from the original design only in the replacement of the original Al foils by Al-coated Mylar foils. The ionization chamber is continually flushed by dry N_2 gas during the time it is being used.

When the intensity distribution of the bombarding particles is homogeneous over the beam cross section, the dose at the position of the ionization chamber is given by (58)

$$D = QWS'_m 10^5/dA\rho \ , \qquad (1)$$

where D is the dose in rads, Q is the charge collected from the chamber, in coulombs; W is the average energy, in eV, required to make an ion pair in the gas used to fill the chamber; S'_m is the mass stopping power of the target material relative to that of the chamber gas; d is separation of the high-voltage foils, in cm; A is the beam area, in cm^2; ρ is the density of the chamber gas at ambient temperature and pressure (ATP), in g/cm^3. The value for W was taken as 34.9 eV per ion pair (59, 60), the value for α particles in dry nitrogen, since values of W and its LET dependence for heavy ions are not known. The mass stopping power of wet tissue relative to dry nitrogen was taken as 1.135 (58). The use of these values with the measured value of the sensitive volume of the ionization chamber and the calculated density of the nitrogen gas at ATP results in an ionization chamber constant of 13.3 krads per μC of charge collected. Figure 4 shows some typical voltage-saturation curves for an ionization chamber with a high voltage-to-collector-foil spacing of 0.82 mm. Curves are presented for different dose rates. This chamber is normally operated at 160 V.

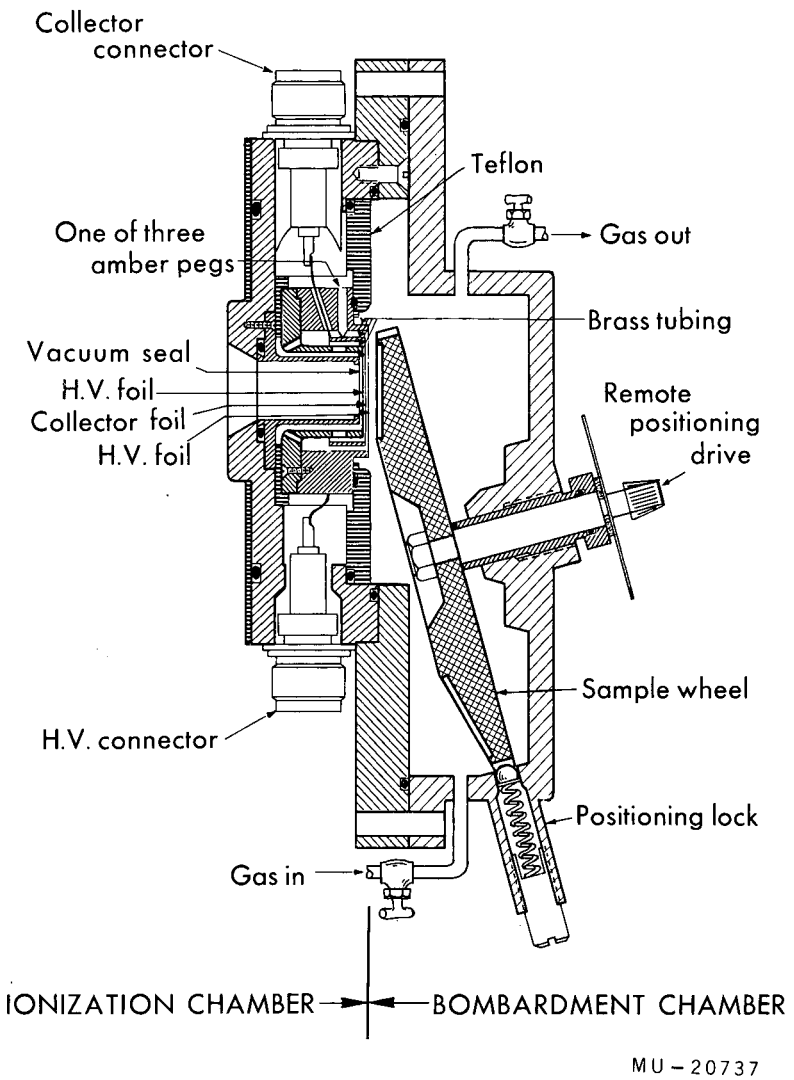

Fig. 3. Ionization chamber and exposure compartment design.

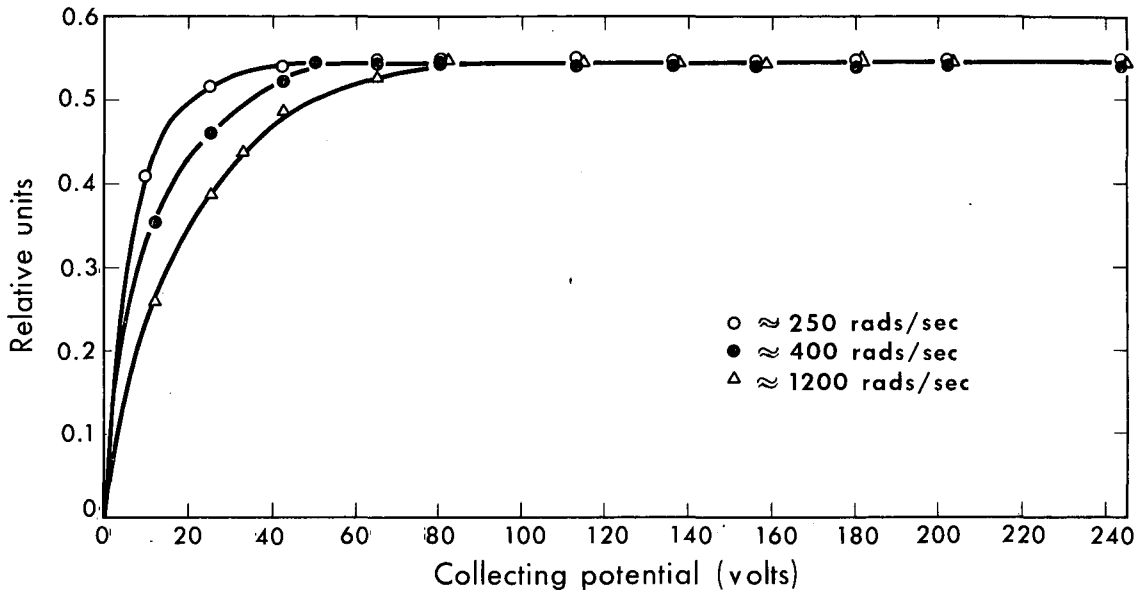

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Fig. 4. Typical ionization-chamber saturation curves for several average dose rates. Spacing between high-voltage foils and the collector foil is 0.82 mm.

With the use of the Al absorbers in the main Faraday chamber, measurements of the external Faraday cup current relative to the monitor foil current can be made. This allows determination of the number-distance curves for the various heavy ions. Figure 5 shows number-distance curves for several heavy-ion beams. If one defines the "mean range" as the absorber thickness that will reduce the number of particles in the beam of 50% of its original value, then estimates of the mean range can be made from these curves. But it must be remembered that the mean range for multi-charged ions is not the absorber thickness that will reduce the ratio of the external Faraday current to that of the monitor to 50% of its original value, since the Faraday current represents the product of the number of particles that reach the cup and the average charge carried by those particles. The mean range for a heavy ion is usually represented by a point located somewhere on the "toe" of the number-distance curve, but its location cannot be determined without information on charge pickup.

If the external Faraday cup is replaced by the ionization chamber, then instead of a number-distance curve, a beam Bragg curve can be determined. Figure 6 shows some Bragg curves obtained with Al absorbers, but corrected for the mass stopping power of tissue relative to that of aluminum in order to present ranges and stopping powers in tissue of several heavy ions.

When a semiconductor detector is placed in the vacuum in a position behind the Al absorbers, it is possible to measure the beam energy. The detectors were silicon surface-barrier detectors, kindly supplied by Dr. Robert Latimer. The theory of operation of surface-barrier detectors has been described in detail by Walter et al. (61). Figure 7 shows a block diagram of the electronic circuits used for pulse amplification and analysis. If a charged particle is totally absorbed within the depletion layer of the detector, an electrical pulse can be measured, which is proportional to the energy of the incident particle. The linearity of pulse height with energy of the incident particle is shown in Fig. 8. If the particle range exceeds the depletion-layer depth,

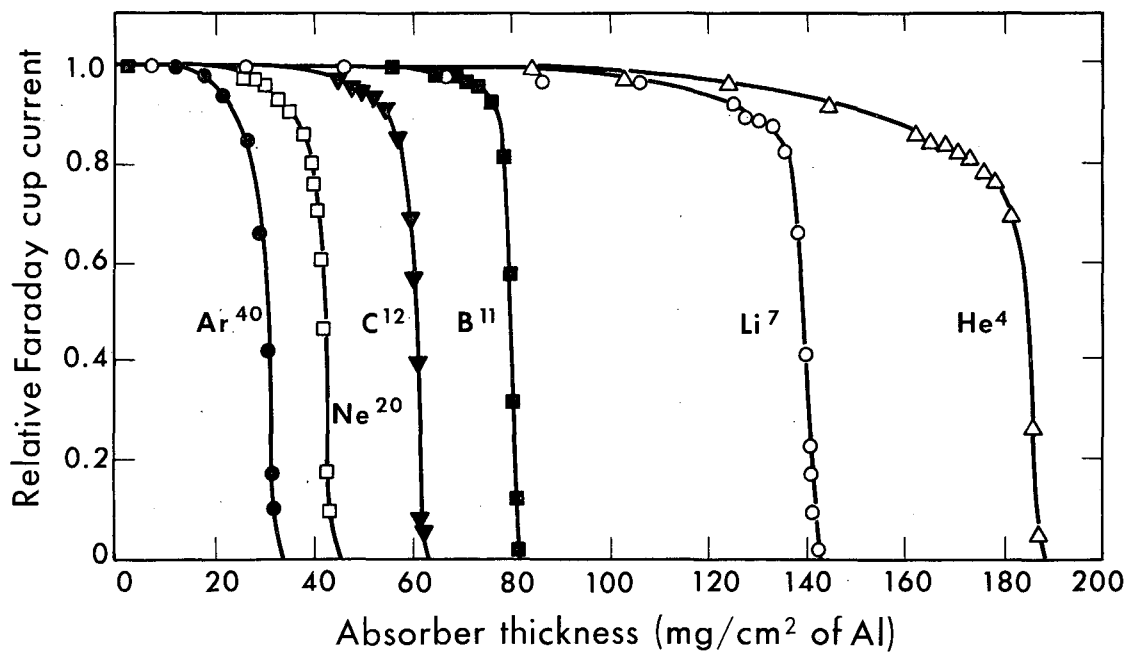

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Fig. 5. Number-distance curves for various heavy-ion beams, with initial energy of 10.4 ± 0.2 MeV/amu.

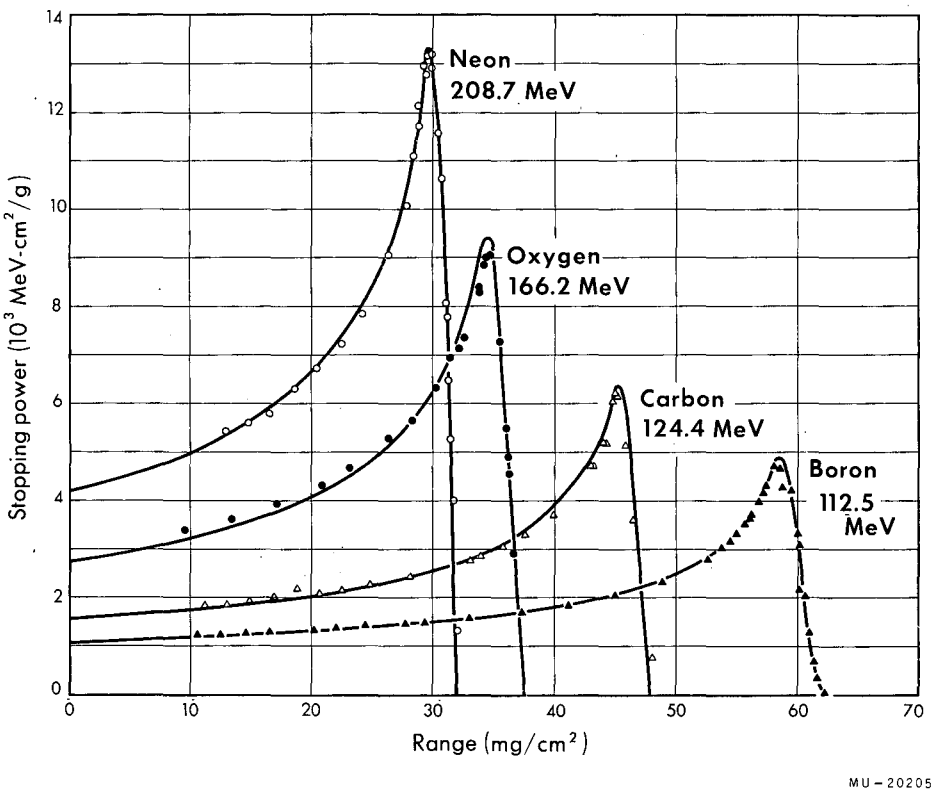

Fig. 6. Bragg curves in tissue of Ne^{20}, O^{16}, C^{12}, and B^{11} ion beams of initial energy 10.4 ± 0.2 MeV/amu. The area under the curves is given.

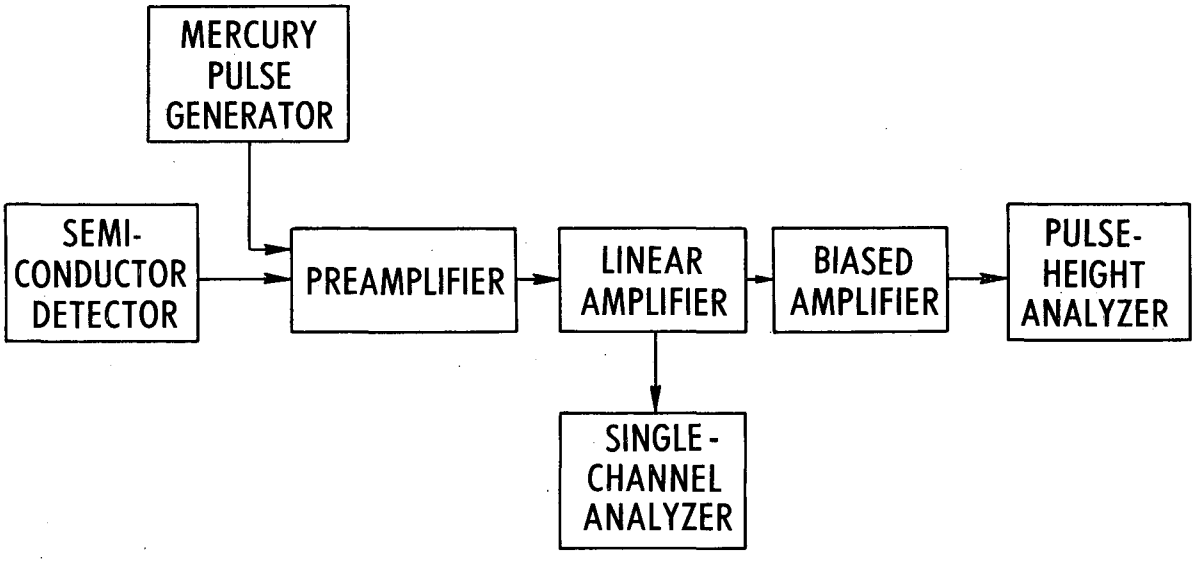

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Fig. 7. Block diagram equipment for measuring beam energy.

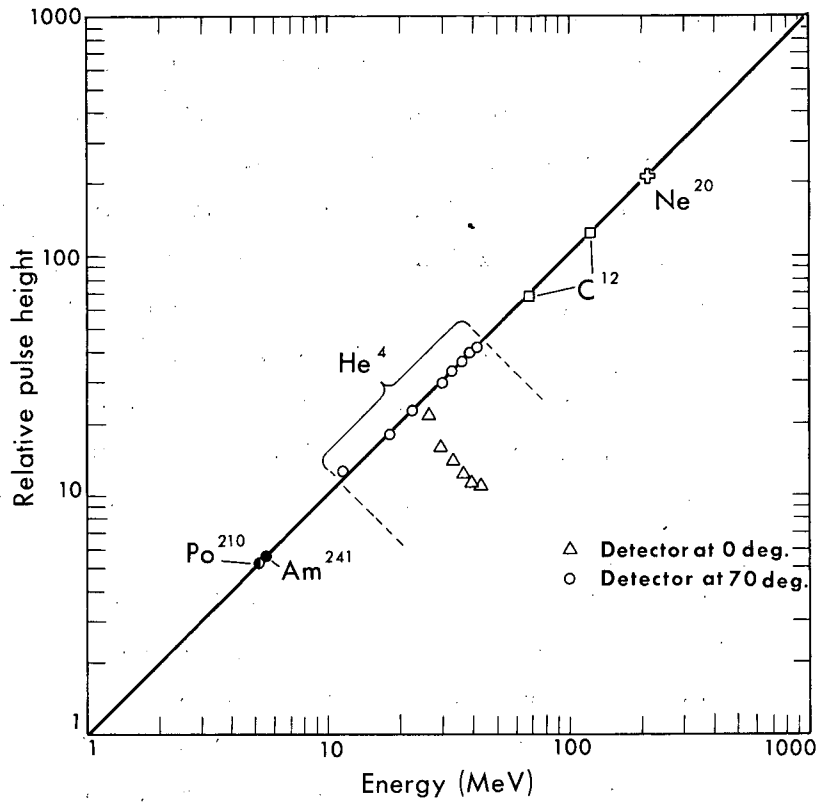

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Fig. 8. Linearity of pulse height with particle energy for heavy ions. He^4 ions with energy greater than 22 MeV will not be stopped within the depletion layer when the ions are incident normal to the surface of the detector, as demonstrated by points (normal incidence) with detector position at 0 degrees.

then the detector is no longer useful for energy measurements, but responds only to energy deposited within the depletion layer. The response is then proportional to dE/dx. The depletion-layer thickness of a surface-barrier detector depends upon the resistivity of the silicon and the applied bias voltage for the detectors used. The depletion-layer thickness for the detectors used was approximately equivalent to the range of a 22-MeV He^4 ion. This is demonstrated by the departure from linearity of the pulse height for higher-energy He^4 ions when they were incident normal to the surface of the detector (Fig. 8).

The effective depletion layer can be increased by allowing the particles to enter the detector at an angle to the normal to the surface. This is demonstrated by rotating the detector 70 deg, so that the particles enter at a 20-deg grazing angle. The detector depletion layer is then able to stop completely the 41.6-MeV He^4 ions.

Beam energy may be determined by use of range-energy tables according to the technique described by Benveniste (62). In this method the energy of the beam is reduced by the addition of aluminum absorbers so that the residual energy of the beam is comparable to that of a known α particle from a radioactive source; then on the basis of the absorber thickness and the range of the α particle, the initial energy of the beam is determined. Figure 9 shows the pulse-height distribution obtained after the full-energy He^4 beam from the Hilac had traversed 176.7 mg/cm^2 of aluminum. The initial beam energy calculated from these measurements is 41.7 MeV, based upon the range-energy data of Williamson and Boujot (63). This is in very good agreement with the expected energy of 41.6 ± 0.8 MeV.

An alternative method of energy measurement is based upon a linear extrapolation from a calibration with the 5.477-MeV α particle from Am^{241}. Figure 10 shows the pulse-height distribution for the full-energy C^{12} beam and its energy as determined by this method. The energy spread of ± 0.04 MeV/amu is much less than the published value of ± 0.2 MeV/amu, which was determined by the average of eleven independent measurements on different occasions (56).

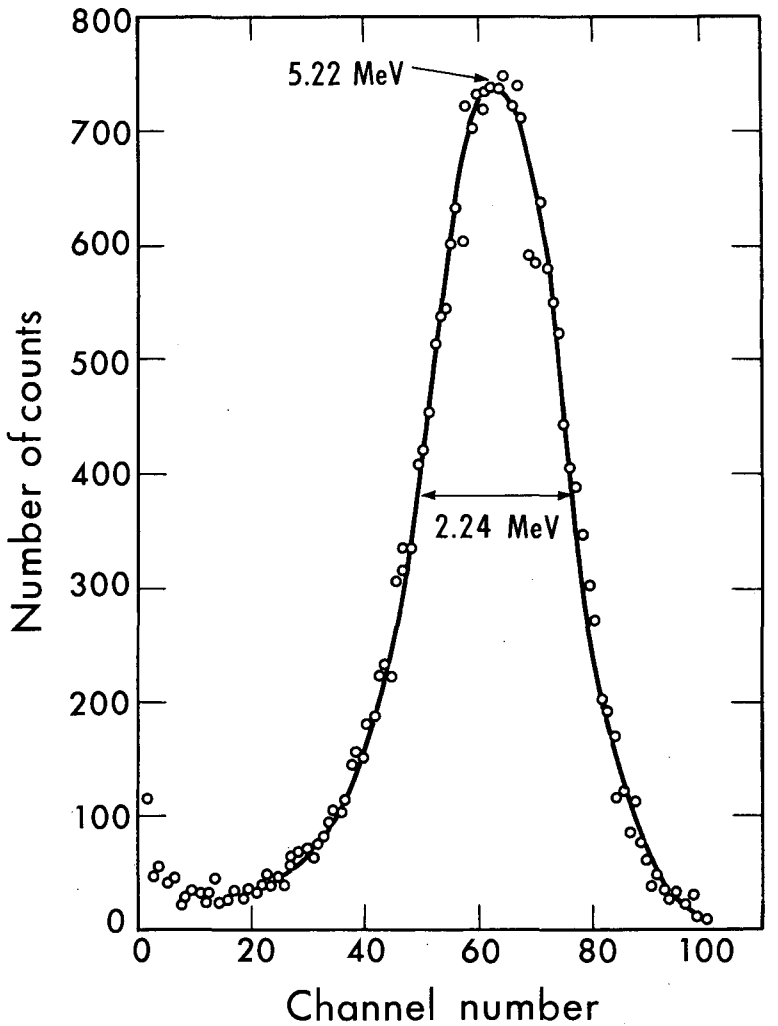

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Fig. 9. Energy of He^4 ions of initial energy 10.4 ± 0.2 MeV/amu after passing through 176.7 mg/cm^2 of aluminum.

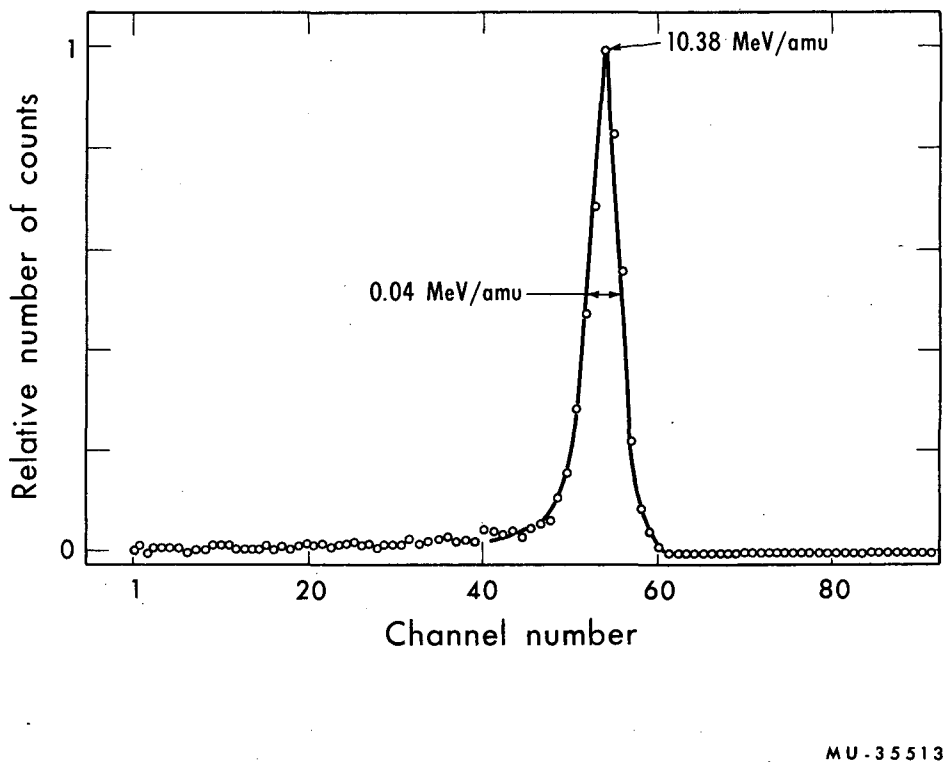

Fig. 10. Energy distribution of full-energy C^{12} ions accelerated by the Hilac.

Figure 11 shows the energy of the C^{12} beam after traversing 39.7 mg/cm^2. The energy of this beam is lower than that used for the biological irradiations, and the energy spread is still less than 3%, which is quite acceptable.

To avoid the long extrapolation from 5 to several hundred MeV, and if range-energy tables are not available, the detector may also be calibrated with the beam on the assumption that the full-energy beam is 10.4 ± 0.2 MeV/amu. Then by inserting appropriate absorbers, one can construct a curve of residual energy versus absorber thickness. Figure 12 shows such a curve for 10.4-MeV/amu He^4 ions. The first derivative with respect to absorber thickness, which gives the stopping power, was obtained by numerical differentiation (solid line). The points along this curve are calculated values of stopping power (63). The agreement with theory is good except in the low-energy region (< 2 MeV/amu), where calculated values are approximately 10% higher than experimental values. In this region the energy straggling is becoming a large fraction of the residual energy and, consequently, energy measurements are becoming uncertain, with a corresponding uncertainty in stopping power. Figure 13 shows the range-energy relation of Ne^{20} ions in aluminum, obtained by transformation of the curve of residual energy vs absorber thickness by subtraction of the absorber thickness from the range of the 10.4-MeV/amu Ne^{20} ion as given by Northcliffe (64). The detector was calibrated with the full-energy beam taken as 10.4 MeV/amu. The solid curve is the range-energy relation for these ions in aluminum, as reported by Northcliffe (64). Data below 1 MeV/amu are uncertain because of the energy straggling. Figure 14 shows the residual energy-absorber thickness and stopping-power relationships for the 10.4-MeV/amu Ar^{40} ions in aluminum. The plotted points along the dE/dx curve represent the mean value for the stopping power (circles) obtained by differentiation of the energy-absorber-thickness curve and relative number of ion pairs collected by the ionization chamber (squares). The solid line is a smooth curve through the experimental points, drawn so that the area under the curve is 413 MeV, which is

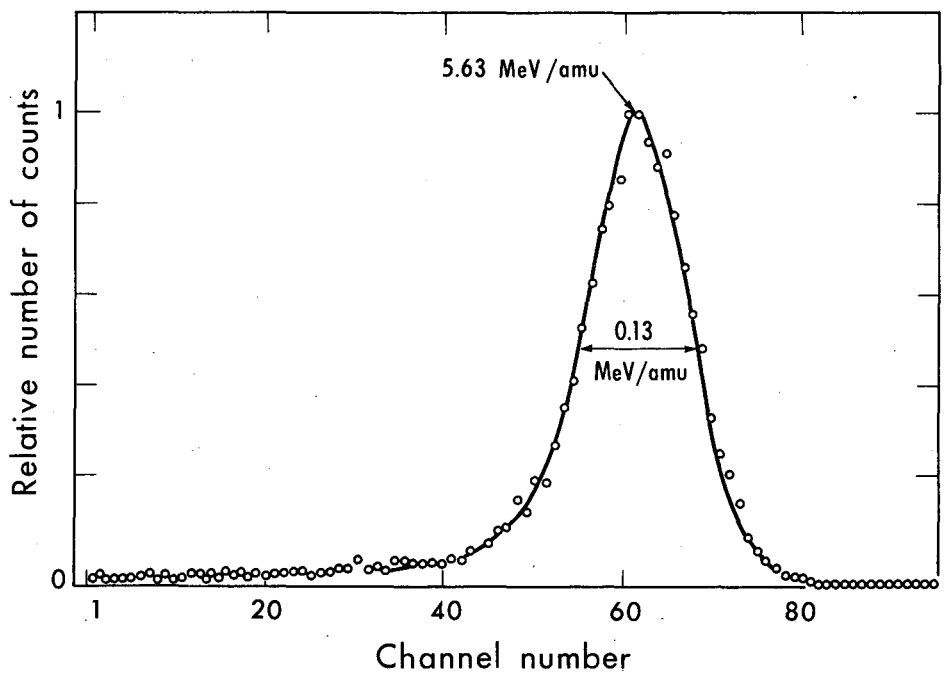

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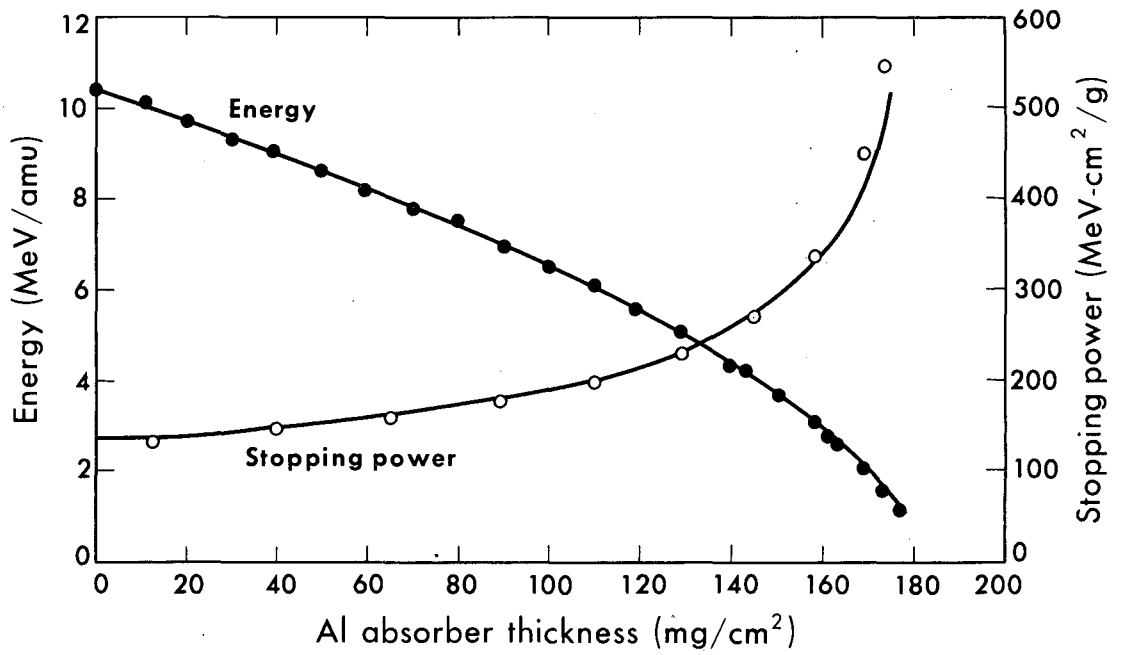
Fig. 11. Energy distribution of C^{12} ions degraded by 39.7 mg/cm^2 of aluminum. Initial energy 10.38 ± 0.04 MeV/amu.



MU-35515

Fig. 12. Residual energy of He^4 ions and stopping power of aluminum as a function of aluminum thickness. Smooth curve of stopping power obtained by differentiation of residual energy curve. Points on stopping-power curve are from Williamson and Boujot (63).

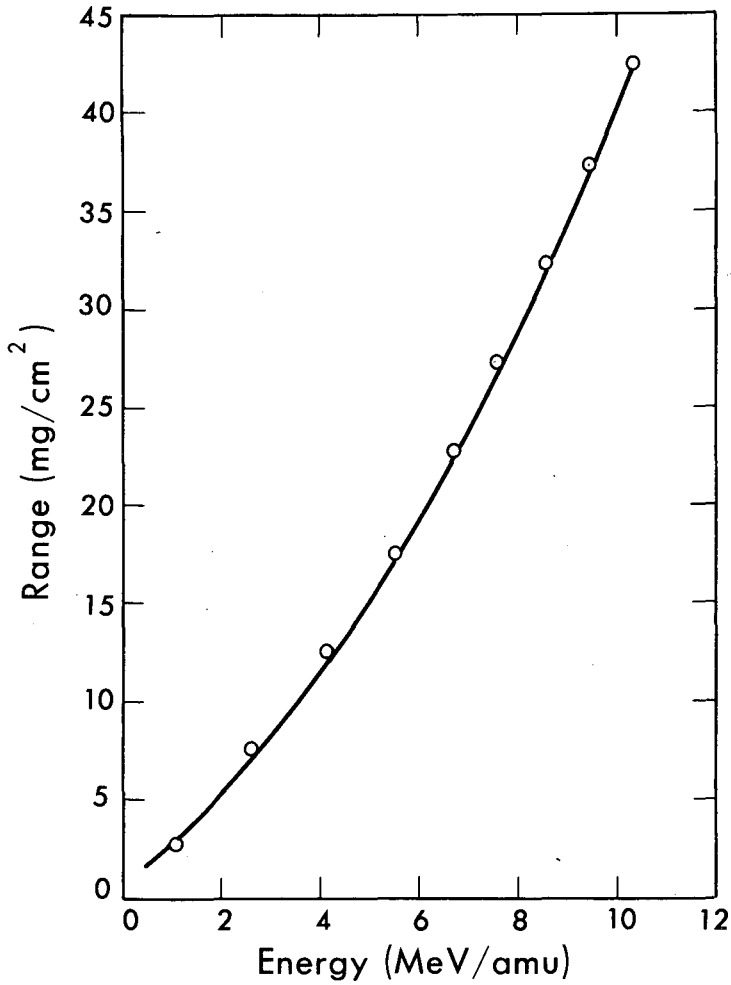


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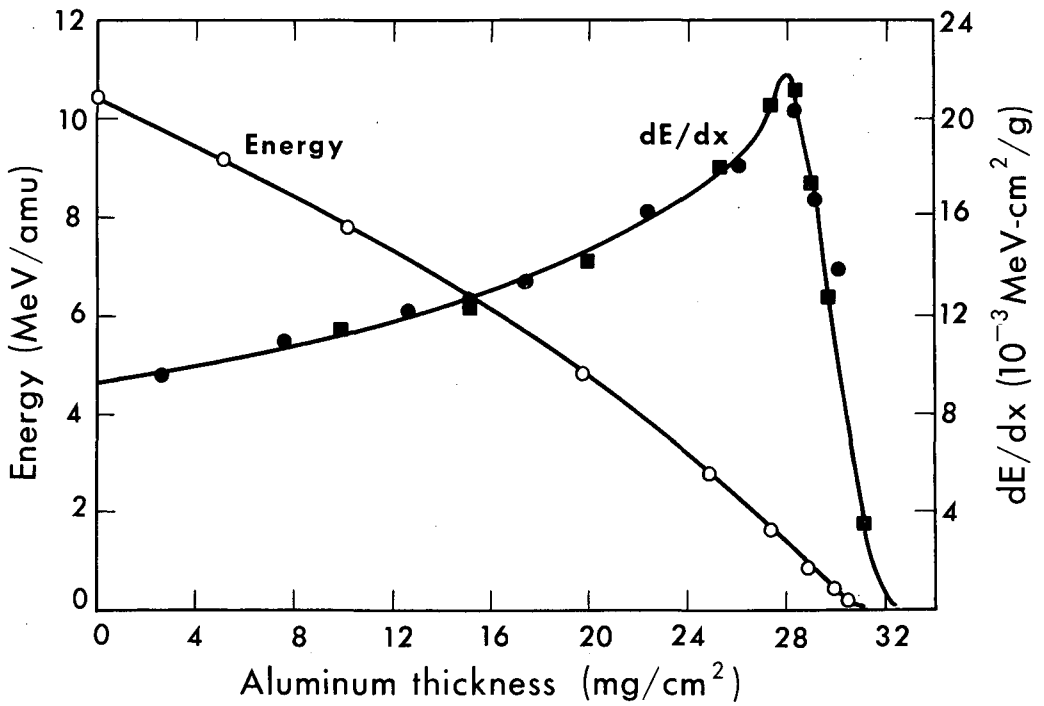
Fig. 13. Range-energy curve for Ne^{20} in aluminum, from Northcliffe, (64). Points calculated from residual energy-absorber thickness relation.



MU-35517

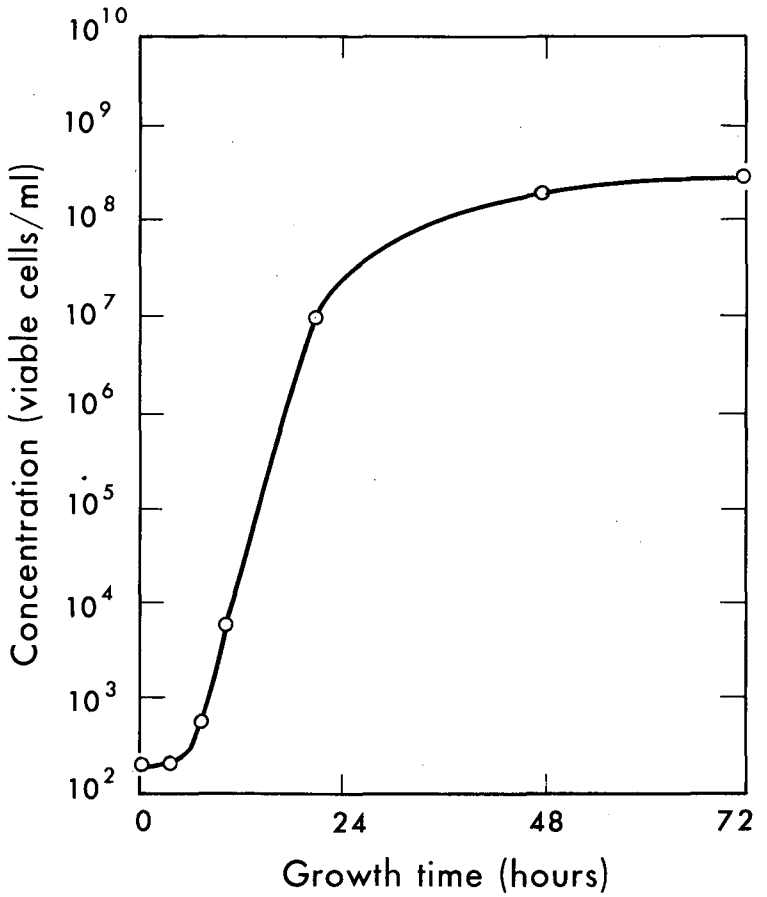
Fig. 14. Residual energy, energy loss, and aluminum absorber thickness relationships for 10.4 ± 0.2-MeV/amu Ar^{40} ions. Energy values (circle) obtained with semiconductor detector; mean energy loss (dot) calculated from energy measurements; relative charge collected by ionization chamber (square). The area under the stopping-power curve is 413 MeV.

within the range of the expected energy value (416 ±8 MeV) for the Ar^{40} beam.

For proper dose measurements, when the ionization chamber is used Formula (1) must be modified to take account of the fact that the sample is placed after the ionization chamber. The correction factor used is the "Bragg ratio" (i.e., the ionization at the sample position relative to that in the middle of the ionization chamber) or the ratio of the stopping power at the sample position to that in the middle of the ionization chamber. By knowing the amount of absorbing material in front of the middle of the ionization chamber and in front of the sample, the correction factor can be determined. In lieu of calculating equivalent thicknesses of Al for all the materials which lie between the vacuum system and the cells, the value for the total equivalent thickness was experimentally determined. Values of residual energy of an Ar^{40} ion beam were determined as a function of the Al absorber thickness with the detector in the Hilac vacuum system. The surface-barrier detector was then placed after the ionization chamber in a location which corresponded to the position of biological samples to be irradiated. The residual energy was again determined as a function of the Al absorber thickness. The path length from the vacuum system to the cells was determined, by the constant difference between these two curves, to be equivalent to 11.0 ± 0.3 mg/cm^2 Al. The nonaluminum absorber in the beam path is not large enough to test the independence of the mass stopping power of the Mylar relative to that of the aluminum from the particle velocity as reported by Schambra (65) and Northcliffe (66).

C. Experimental Method

Yeast was subcultured from YEPD slants onto YEPD plates. A portion of a single-cell isolate from the subculture plate was used to inoculate 10 ml of double-strength YEPD liquid media, which was contained in a 50-ml DeLong culture flask with a metal closure. The flask was shaken on a vibrating shake table which was within a 30° incubator. After 48 hours' growth, the cells are in a stationary growth phase (Fig. 15); the cells were washed three times with double-distilled,



MU-35518

Fig. 15. Growth curve of strain X481 in double-strength YEPD liquid media at $30 \pm 0.6\,^{\circ}C$.

de-ionized water and were resuspended in double distilled, de-ionized water to make a stock solution. Microscopic examination of this stock solution usually revealed less than 3% budding cells, no asci, and no clumps.

Fifty-λ aliquots of the stock solution were placed on sterile 13-mm-diameter membrane filters (Millipore, type HA) which had previously been affixed at the margins, with Eastman 910 adhesive, to 13-mm-diameter absorbent pads. The water was quickly absorbed into the pad, leaving the cells in a monolayer on the surface of the filter. The pad was then saturated with distilled water. Because of the high porosity of the filter, the cells remained in contact with the water, but the thickness of the layer of water covering the cells was negligible. The cells were then exposed to the radiation in an air atmosphere. Immediately following irradiation, the absorbent pad was discarded and the membrane filter was dropped into a test tube containing 5 ml double-distilled, de-ionized water. After the cells had been resuspended from the filter, small portions were removed and appropriately diluted with distilled water so that a 0.2-ml aliquot would contain between 100 and 200 viable cells; these aliquots were spread on YEPD plates. The remainder of the original suspensions was transferred, without the membrane filters, to 50-ml DeLong culture flasks with metal closures. The flasks were shaken with the vibrating shaker within a 30°C incubator. The suspensions were stored in the dark for periods up to and including 4 days. At various times aliquots of the suspensions were again diluted and plated.

All plates were incubated at 30°C and were counted several times before a final count was obtained, which was at least 5 days after the cells had been spread on the plates. The spreading of the cells on the plates immediately after irradiation will be referred to as "immediate plating." Platings done after incubation of the suspensions will be referred to as "delayed plating."

All glassware used in these experiments, except the dilution tubes, had been immersed in distilled water for at least 24 hours prior to heat sterilization. This procedure was used in an effort to remove possible trace contaminates which can inhibit recovery (37).

III. EXPERIMENTAL RESULTS AND DISCUSSIONS

A. Heavy-Ion Irradiations

Since there is considerable belief that there is a greater amount of irreversible damage to DNA following heavy-ion and fast-neutron irradiations than following x irradiations, samples of strain X841 were exposed to graded doses of several of the heavy-ion beams produced by the Hilac and to 50-kV x rays. The results are shown in Figs. 16 through 18. The energy of the heavy ions in the samples and the stopping power of tissue for these ions are given in Table I. The survival

Table I. Energy and stopping power for some heavy ions.

Radiation	Energy (MeV/amu)	Stopping power in tissue ($MeV\text{-}cm^2/g$)
He^4	9.9	180
C^{12}	9.1	1765
Ne^{20}	8.2	4920

curves of the delayed platings have been compared with the curves of the immediate plating by means of a simple dose-modifying factor (DMF), since a consistent variation in the intercept has not been observed. The DMF values for the experiments reported in Figs 16 through 18 are shown in Table II. Values of the DMF for x irradiations have varied

Table II. Dose-modifying factors as found in irradiation of yeast strain X841.

Radiation	DMF
x ray	1.9
He^4	2.1
C^{12}	2.3
Ne^{20}	1.9

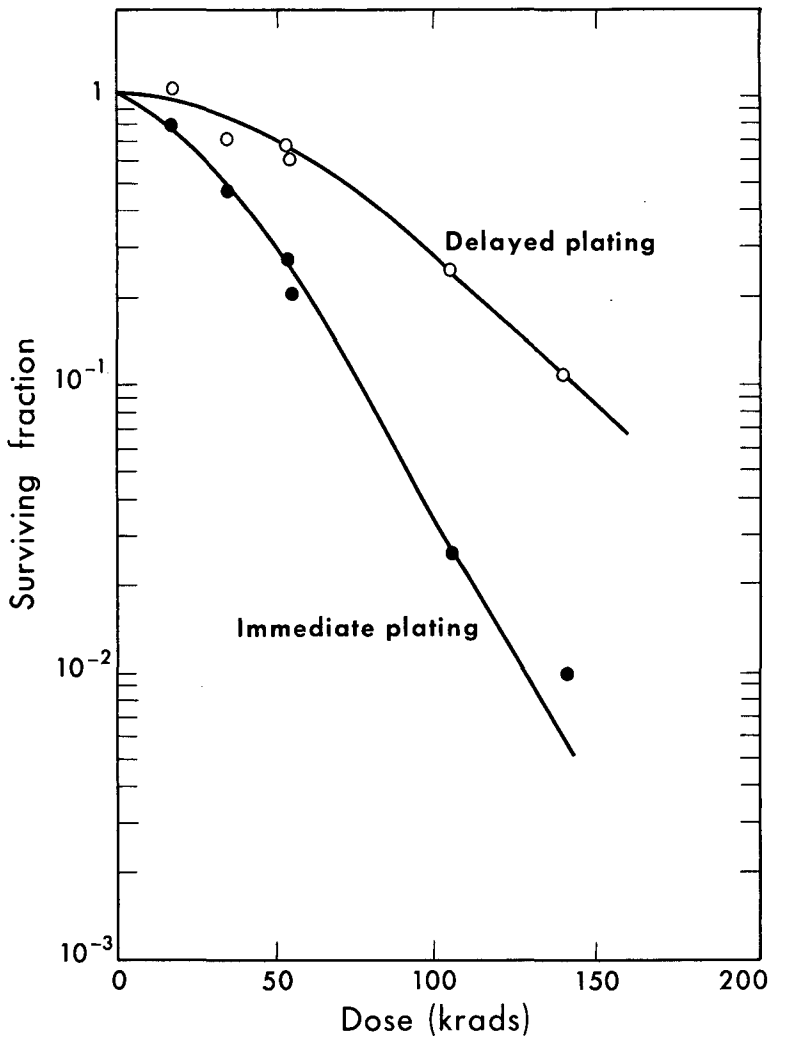


Fig. 16. Immediate and delayed (48 hours) platings of X841 following 50-kV x irradiations.

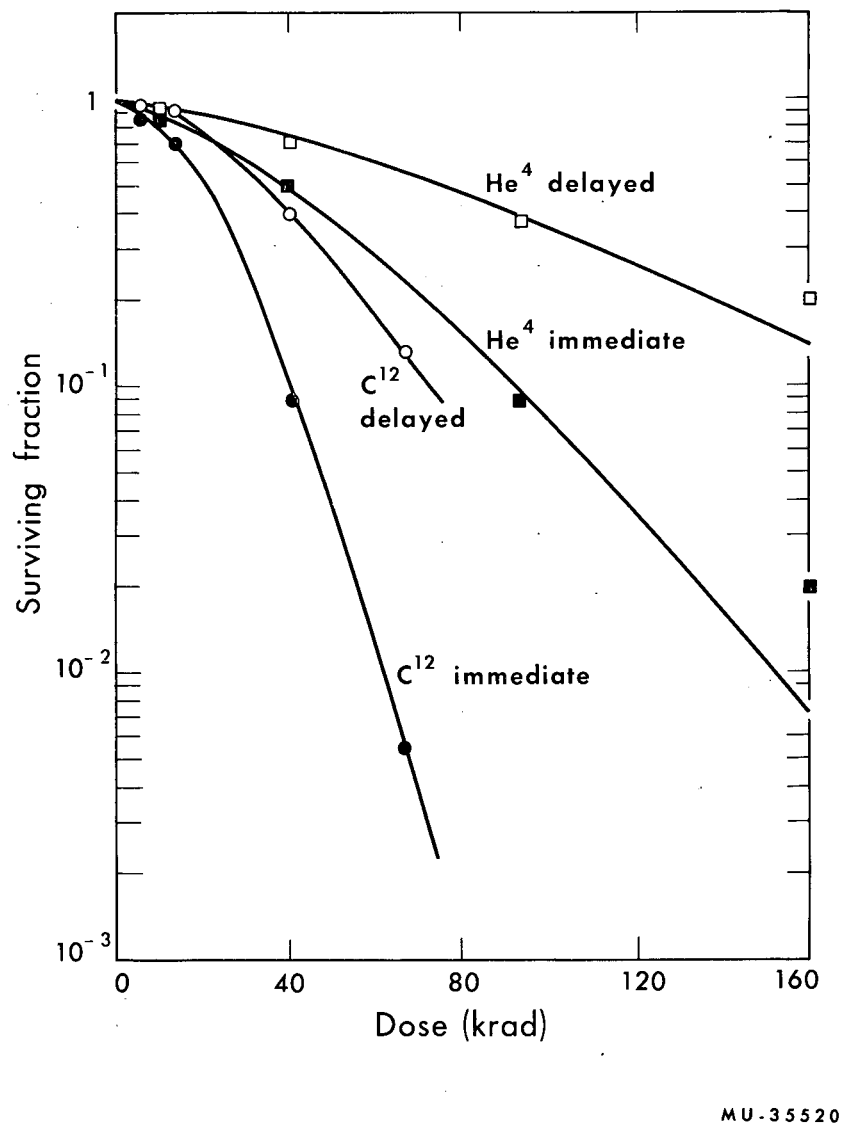


Fig. 17. Immediate and delayed (48 hours) platings of X841 following heavy-ion (C^{12} and He^4) irradiations.

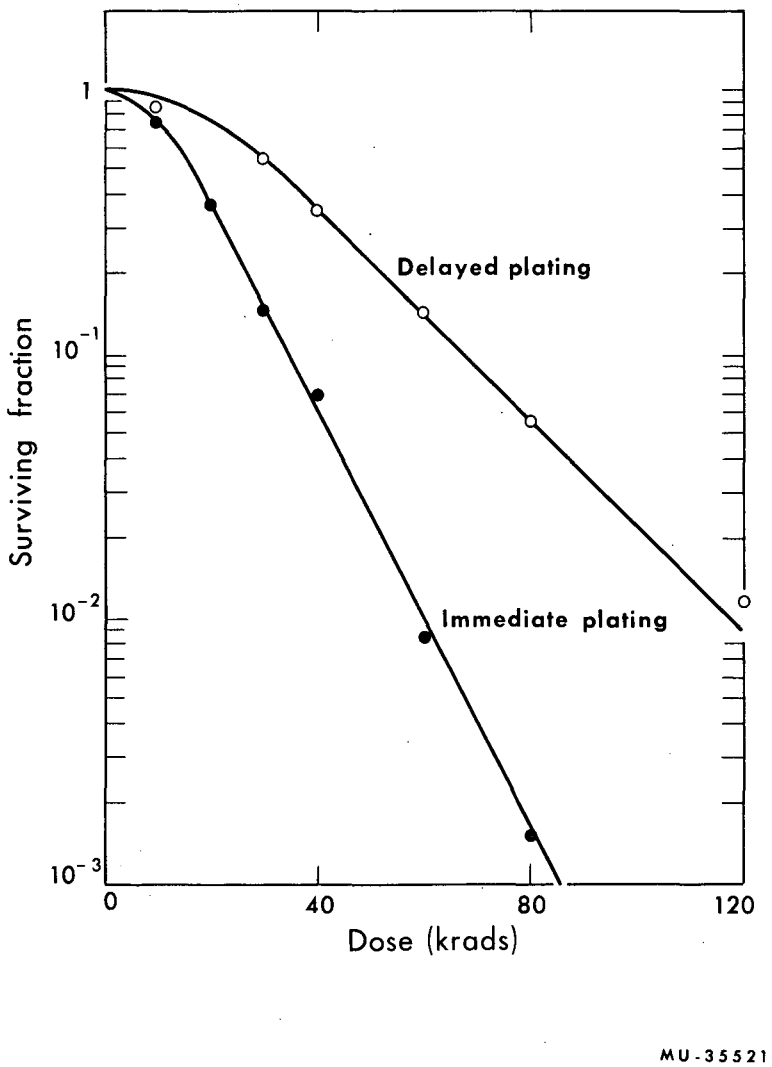


Fig. 18. Immediate and delayed (48 hours) platings of X841 following Ne^{20} irradiation.

from 1.6 to 2.7 on different occasions, but they have usually been very close to 2.0. Because of the variability observed in the DMF, it is felt that the values in Table II are not significantly different from 2.0; therefore, recovery following all heavy ions used, even the densely ionizing neon ions, is not significantly different from that observed following x irradiations.

Recovery has also been observed following x-ray inactivation under both aerobic and anoxic conditions (27, 32, 37), and within the experimental error the recovery DMF is probably the same. Thus, it appears that the recovery mechanism operates equally well on both the oxygen-dependent and the oxygen-independent components of radiation damage. The heavy-ion results suggest several possibilities: (a) that the damage caused by heavy ions in yeast cells is similar to that caused by x rays; (b) that the recovery mechanism does not actually repair the lesions but by-passes the lesion (by recombination or de novo synthesis of new DNA); (c) or that lesions are, in fact, repaired and that the recovery mechanism is capable of repairing the presumed massive lesions caused by heavy ions.

B. Glycerol Protection

The independence of the recovery DMF of the stopping power is reminiscent of the experiments by Manney et al. (44) on glycerol protection of haploid yeast. In those experiments the glycerol protection was found to be nearly independent of LET.

Further experiments were done to see if glycerol could also protect diploid yeast and to see if recovery were possible following glycerol protection.

Cells of strain X481 were equilibrated with 6 M glycerol for 1 hour prior to being placed on the membrane filters; the absorbent pad was saturated with 6 M glycerol. Cells from the distilled water suspension were irradiated and plated in parallel with glycerol-treated cells. Both groups of cells were resuspended in distilled water prior to plating.

Figures 19 and 20 show the results of an x-ray and a He^4 irradiation. Table III presents the value of the DMF obtained both with and without the glycerol for both radiations. The differences observed are not significant, and this result is taken as evidence that the recovery mechanism operated independently of the glycerol protection mechanism.

Table III. Effect of glycerol on dose-modifying factors.

	Without glycerol	With glycerol
x Ray	2.2	2.4
He^4	2.6	2.3

C. Recovery and Respiratory-Deficient Yeast

Respiratory-deficient yeast arises spontaneously from normal cells. These mutants are recognized by their colony morphology when plated on solid media (53). These "vegetative petite" mutants have been shown by Ephrussi (67, 68) to be cytoplasmic mutants probably arising from irreversible loss or inactivation of self-reproducing cytoplasmic units which are necessary for the synthesis of cytochrome oxidase and other enzymes.

Patrick et al. (31) have reported that storage of irradiated normal diploid suspensions leads to an increase in the absolute titer of both respiratory-deficient and normal colonies, but to a reduction in the ratio of respiratory-deficient to normal colonies at all dose levels. These authors concluded that either recovery occurs preferentially in cells containing no damage relevant to petite formation, or damage relevant to petite formation may be reversed by prolonged postirradiation incubation in distilled water, or both.

A petite mutant of X841 was isolated and tested for its respiratory deficiency by failure of growth on PET media (see page 5), and by the "tetrazolium overlay test" (69). This petite strain was then

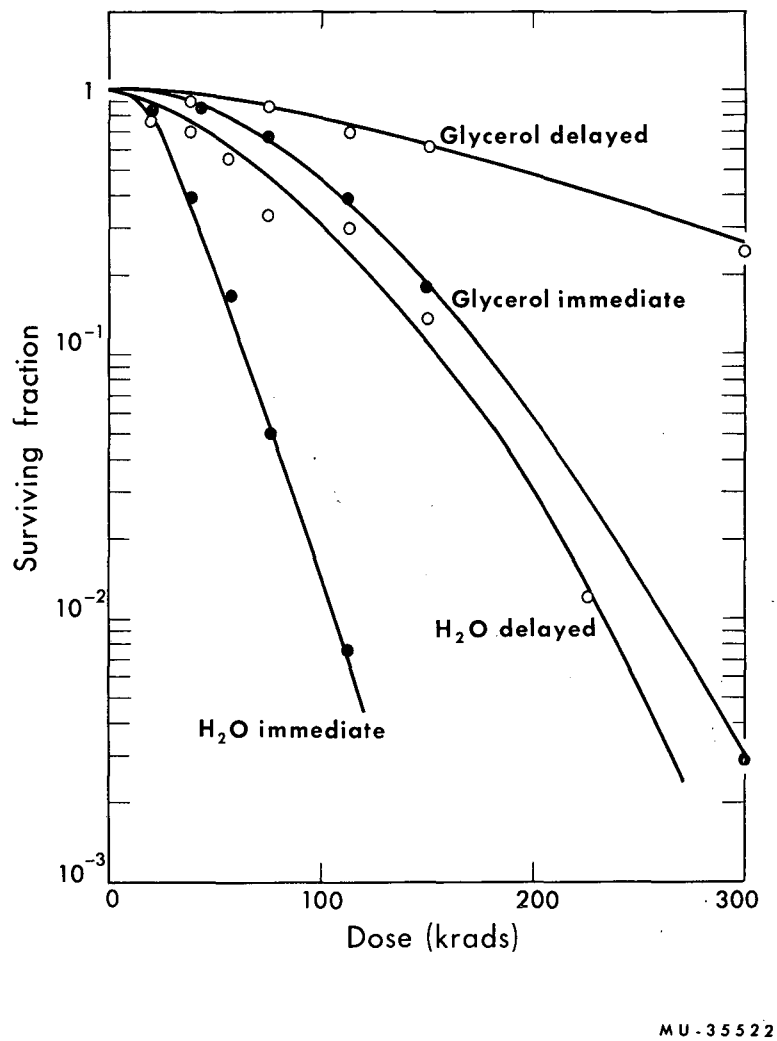
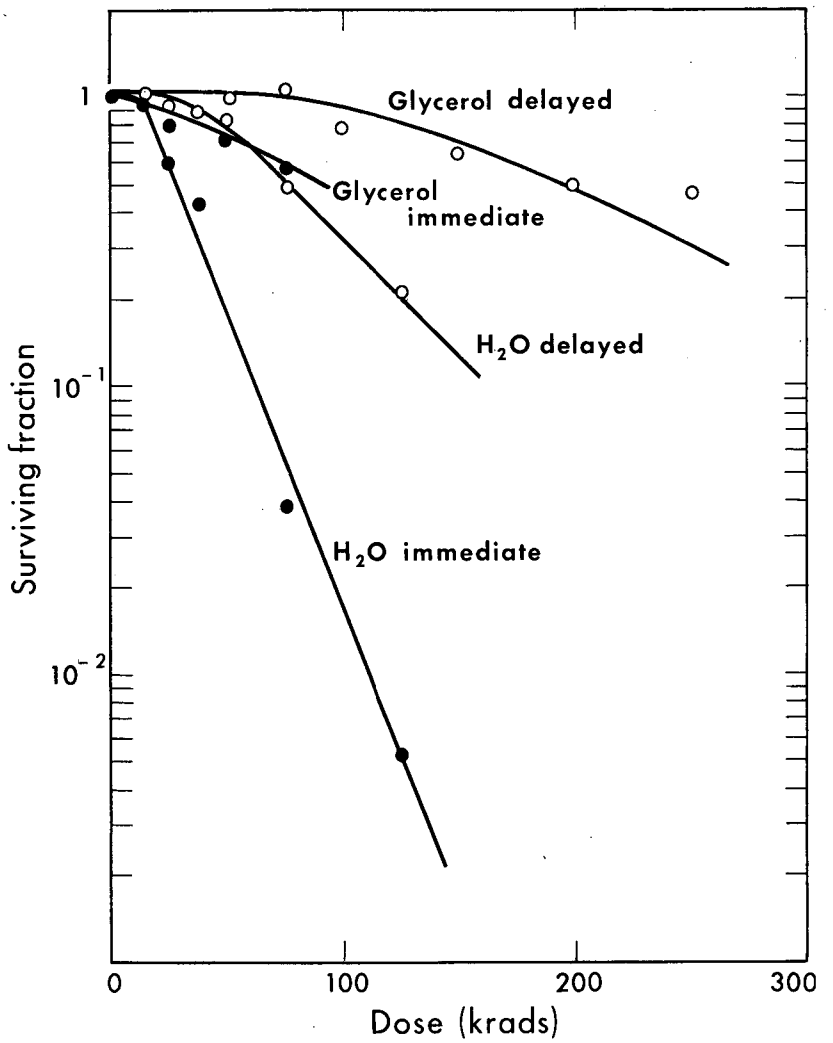


Fig. 19. Immediate and delayed (48 hours) platings of X841, both with and without 6 M glycerol protection during irradiation with 50-kV $\bar{x}$ rays.



MU-35523

Fig. 20. Immediate and delayed (48 hours) platings of X841 with and without 6 M glycerol protection during 9.9-MeV/amu He^4-ion irradiation.

tested for its ability to recover from x radiation under the same conditions as used for strain X841. Table IV shows the number of viable cells per milliliter as a function of the absorbed dose and the storage time. The data indicate that the respiratory-deficient mutants are not capable of reducing the lethal damage caused by x rays when incubated in distilled water, and, in fact, many cells that have sustained sublethal damage are not able to survive the postirradiation storage treatment, which does not noticeably affect the control population. These results confirm the work of Rmezova and Tret'iakova (28), who reported similar findings with a petite strain of Saccharomyces vini.

Table IV. Number of viable cells per ml of the respiratory-deficient mutant of strain X841.

	Dose in krads				
Hours	0	10	25	50	75
0	1.71×10^6	1.75×10^6	1.3×10^6	3.35×10^5	1.14×10^5
24	1.77×10^6	1.43×10^6	---	2.88×10^5	5.61×10^4
48	1.76×10^6	1.41×10^6	7.91×10^5	1.54×10^5	1.74×10^4

D. Relation to Sporulation

The failure of haploids and petite diploids to recover (28, 36, 37), and the recovery of diploid and higher ploidies (36, 37) following ionizing radiations, suggest the possibility of a common pathway shared by sporulation and recovery processes. This possibility is further supported by microscopic examination of cell suspension that have been stored for several days at 30°C in distilled water. Under these conditions, which have been utilized to elicit recovery, asci have been observed. Recovery cannot be explained as being due to sporulation, since recovery is possible in the absence of oxygen during the storage period (25, 29, 32, 37) and is also enhanced by 100% oxygen (25, 29, 37), while sporulation requires the respiration of oxygen and is also inhibited by oxygen concentrations above 40% (70).

The ability of normal diploid yeast (X481) to recover was tested when the cells were stored, suspended in a dextrose-phosphate buffer (0.5% dextrose, M/15 phosphate, pH 6.4). Low concentrations of dextrose are known to stimulate sporulation while concentrations between 0.33 and 1.0% are inhibiting (71, 72). Figure 21 shows the results of immediate and delayed platings following 50-kV x rays. Recovery was not inhibited by this concentration of dextrose, and this is further indication that the two processes are not identical.

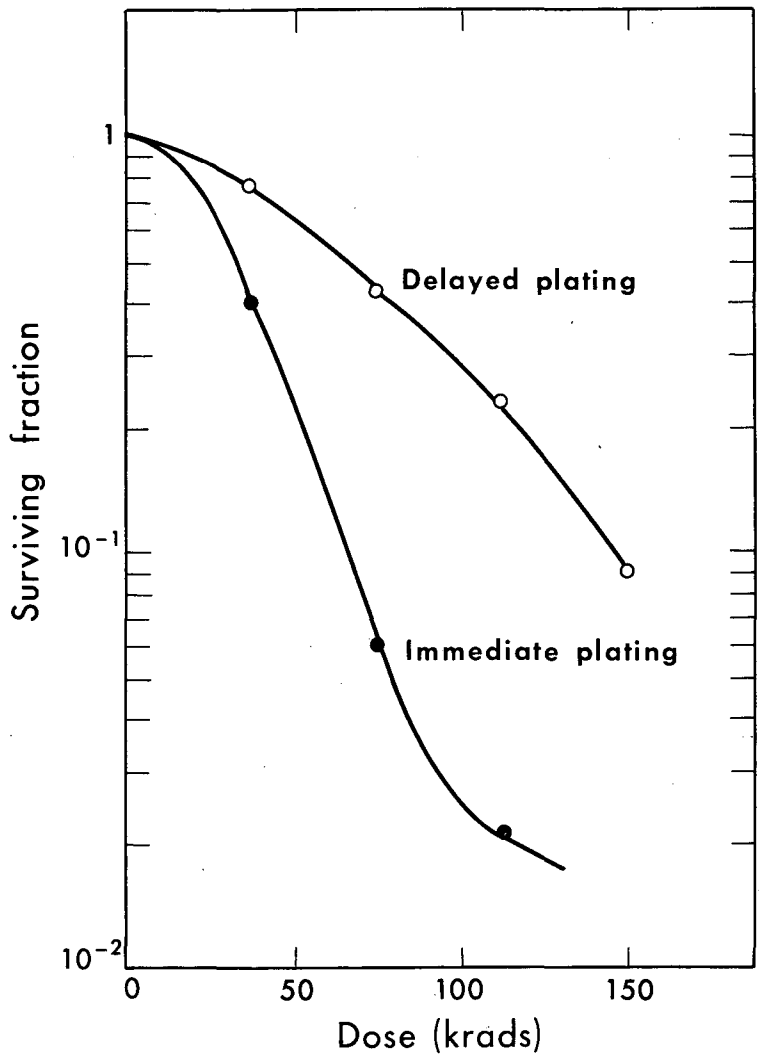
Sporulation is also known to be dependent on cell concentration. The optimum concentration is about 2×10^6 cells/ml (68). Recovery has shown no such concentration dependence as shown in Table V. Korogodin et al. (25) have reported a concentration dependence, but only at high doses when comparing dilute 10^4/ml suspensions with packed cells (10^{10}/ml).

Table V. Percent survival following 45 krads 50-kV x-ray irradiation and storage in distilled water at 30°C for various periods.

Cell concentration (cells/ml)	Time (hours)			
	0	2	8	19
2.1×10^6	47.3	51.0	59.3	75.1
4.1×10^4	47.3	---	63.9	76.6
7.9×10^2	47.3	51.6	62.8	77.5

It is known that homozygosity for mating type leads to inability to sporulate (73) and to a greater radiation sensitivity (9, 74). x-Ray irradiation of XJ4 (αα-mating-type constitution) and subsequent recovery in distilled water have been observed; the results are shown in Fig. 22.

The radioresistant portion of the survival curves is due to the presence of a large fraction of budding cells which have a higher radioresistance than cells that are not budding (75). Haemocytometer counts revealed that during the storage period neither the total number of cells



MU-35524

Fig. 21. Immediate and delayed (24 hours) platings of X841, following 50-kV x rays. Cells were stored in dextrose-phosphate buffer (0.5% dextrose; M/15 phosphate; pH 6.4).

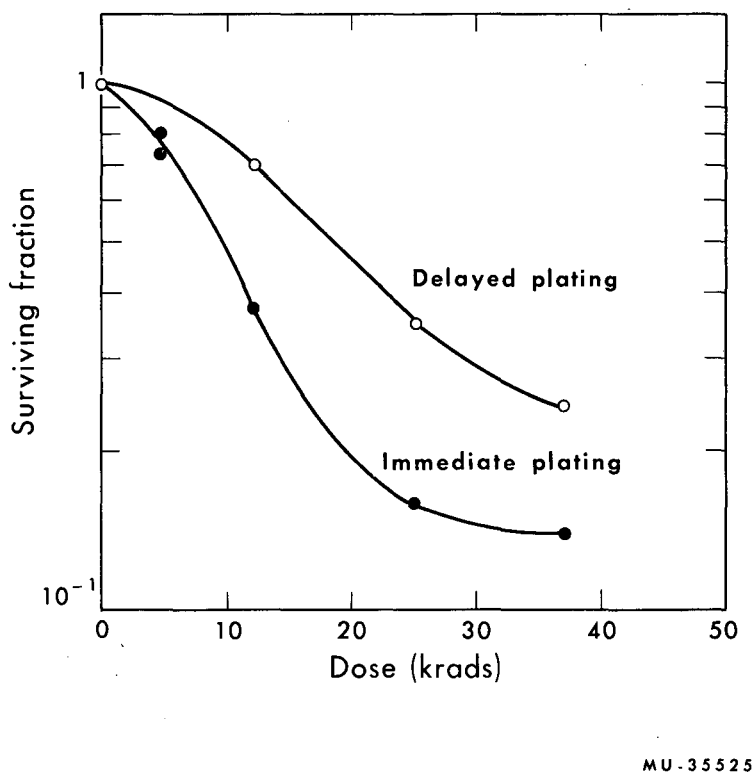


Fig. 22. Immediate and delayed (24 hours) platings of XJ4 following 50-kV x rays.

nor the budding fraction changed. In spite of this large component of radioresistant cells, the DMF was the same as found with strain X841, that is, 2.0. An attempt was made to decrease the budding fraction of the initial population by using cells which had been allowed to grow for 10 days on a YEPD plate. This technique has been used to decrease the radioresistant component of populations of haploid cells (76). The results of the immediate and delayed platings of these "starved" cells of strain XJ4 are shown in Fig. 23. The DMF is 1.5, which is lower than normally encountered, but is consistent with the results of Karabaev (31), who found that recovery is dependent upon the physiological state of the cells, and that starvation leads to a decrease in the ability of the cell to recover. The results of the experiments with strain XJ4 are further proof that sporulation per se is not the critical step in recovery. These results also indicate that heterozygosity of the mating-type allele is not required for a recovery mechanism to be operative.

E. Allelic Recombination Frequencies

With strain X841, changes in the frequency of -ar revertants due to allelic recombination (77) at the ar_4 locus have been observed following storage of unirradiated cells in distilled water, phosphate buffer, or 0.3% potassium acetate sporulation media. Cells of X841 were harvested from a 48-hour YED liquid culture, washed three times with M/15 phosphate buffer (pH 6.0) and resuspends at a concentration of 10^6/ml in M/15 phosphate buffer with 0.3% potassium acetate. The cells were shaken and stored at 30°C. At various intervals, aliquots were withdrawn and appropriate dilutions were plated on YED, -ar, and M-VIT supplemented with tryptophan methionine, and histidine. The difference between the -ar and the supplemented M-VIT media is that the -ar media contains five additional amino acids (adenine, leucine, lysine, threonine, and uracil). The -ar media is used to determine the total number of -ar revertants (both haploid and diploid), while the supplemented M-VIT can be used to discriminate against haploid -ar revertants (there is a 3% probability that a haploid -ar revertant will

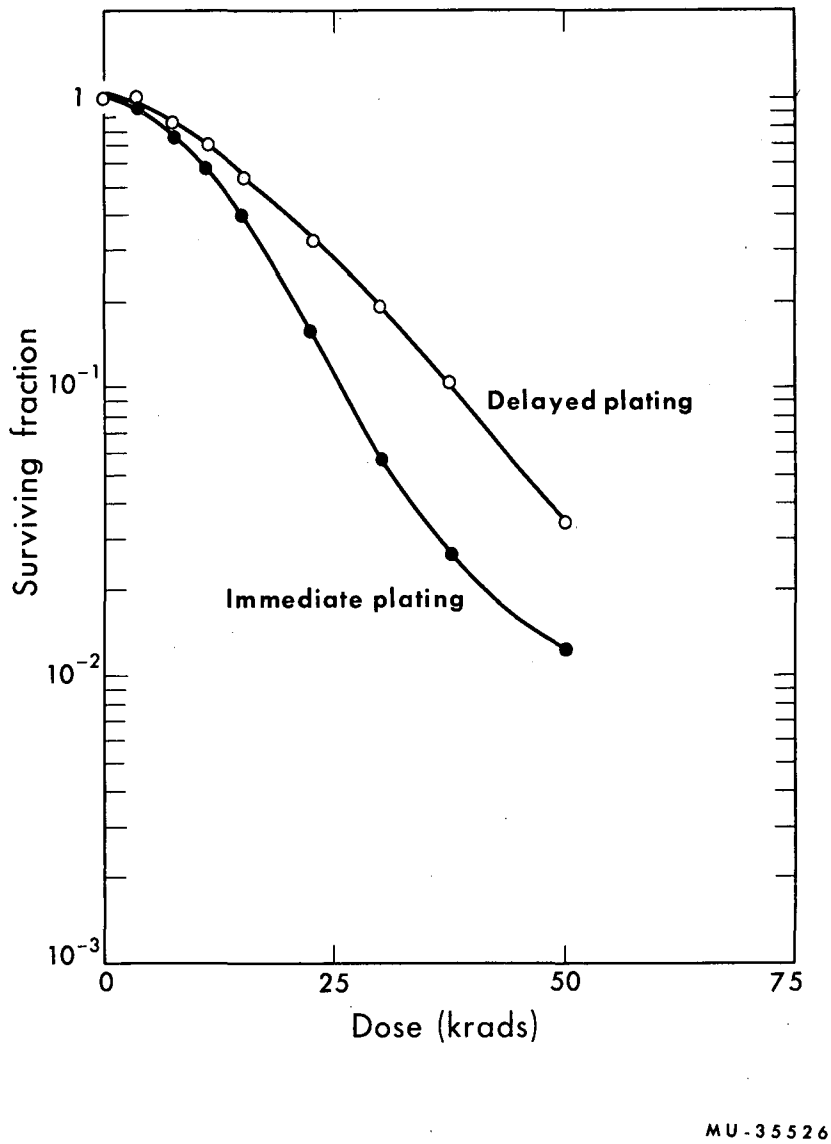


Fig. 23. Immediate and delayed (24 hours) platings of "starved" XJ4 following 50-kV x rays.

grow on the supplemented M-VIT). The increase in frequency of -ar revertants due to storage is shown in Fig. 24. The ratio of the frequency of revertants on supplemented M-VIT to that on -ar (Fig. 25) represents the fraction of revertant colonies that are diploid. The solid line is a smooth, monotonically decreasing curve connecting the experimental points. The frequency of colonies on YED with the typical red colony color resulting from homozygosity or hemizygosity of the ad_2 gene is shown in Fig. 26. The solid curve connecting the points is drawn so that the curve is linearly related to the fraction of -ar revertants that are haploid, derived from Fig. 25, since the fraction of red colonies represents the fraction of cells committed to meiosis (77).

During the first 6 hours in the potassium acetate media, the cells remain uncommitted to sporulation, but after that time the cells start becoming committed to sporulation. By 24 hours most of the cells that will ultimately sporulate have become committed to that course.

If the cells are irradiated with 50-kV x rays (75 rads) before storage in the acetate solution, then an induced allelic recombination (73, 78) is observed as well as recovery. Recovery of viability begins (Fig. 27) promptly after irradiation, and is maintained at a maximum rate for the first 8 hours, then the rate decreases as recovery nears completion at 16 hours. Revertant colonies were observed on both the -ar and the supplemented M-VIT media. During the first 8 hours of storage, while recovery rate is maximum, the revertant frequencies on both types of media are decreasing (Fig. 28). This indicates that damage associated with allelic recombination is being repaired. The ratios of revertant colonies on supplemented M-VIT to those on -ar is shown in Fig. 29. Initially, only 36% of the cells that produced revertant colonies on -ar were capable of producing colonies on supplemented M-VIT. This increases to 80% after 24 hours. Colonies on the immediate (0-hour) -ar plates were tested by replica plating (79) for nutritional requirements that could account for the differences observed because of the difference in plating media. It was found that 90% of the

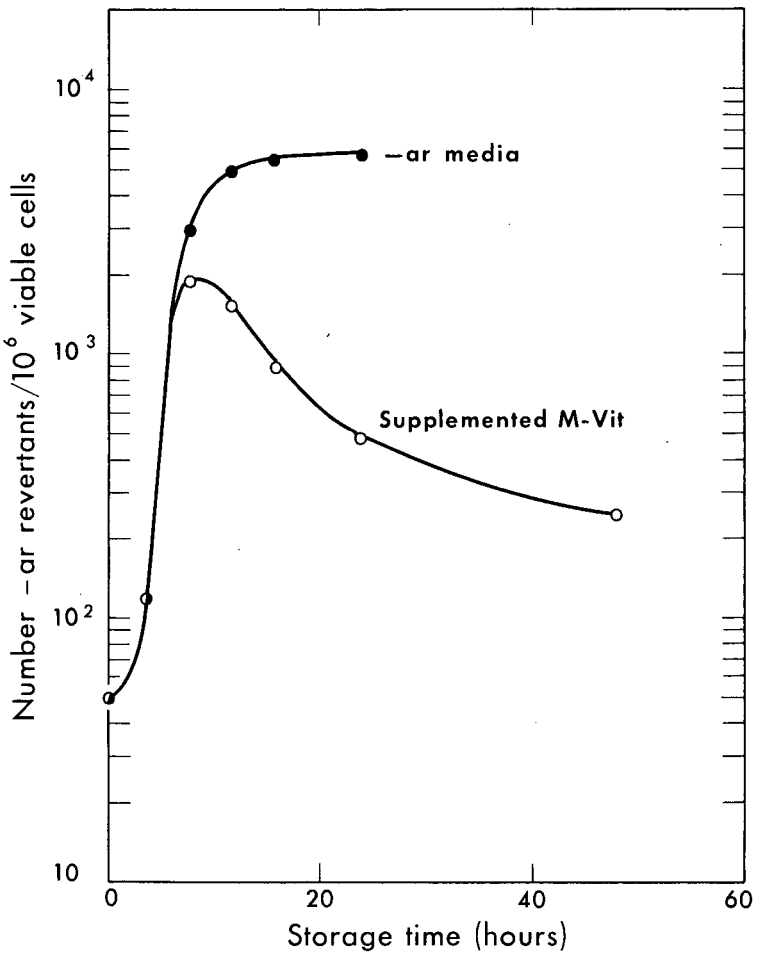


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Fig. 24. Allelic recombination frequencies of unirradiated X841 cells after various periods in 0.3% potassium acetate in M/15 phosphate buffer (pH 6.0).

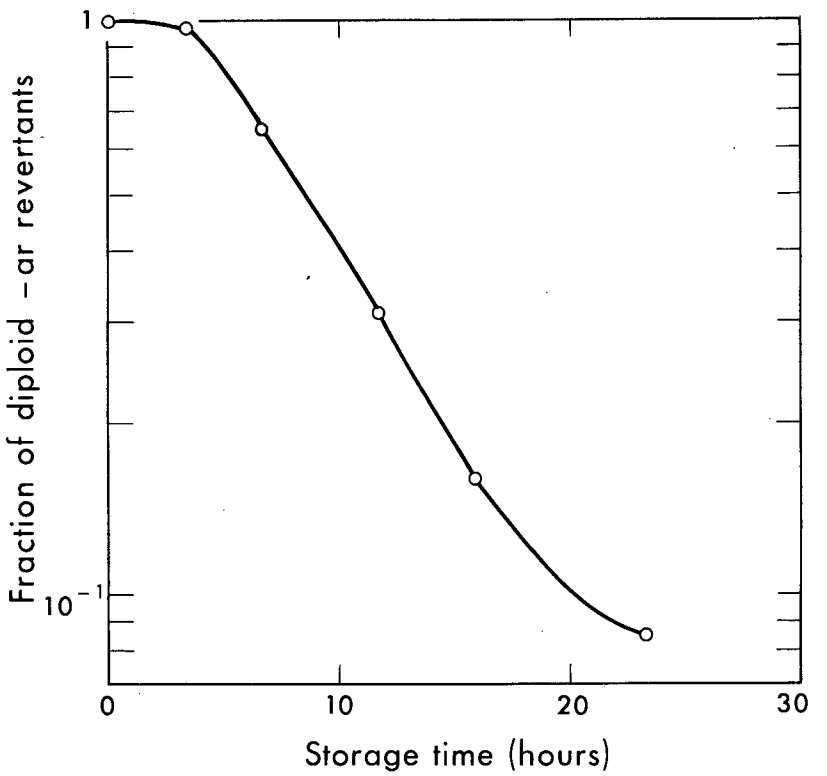

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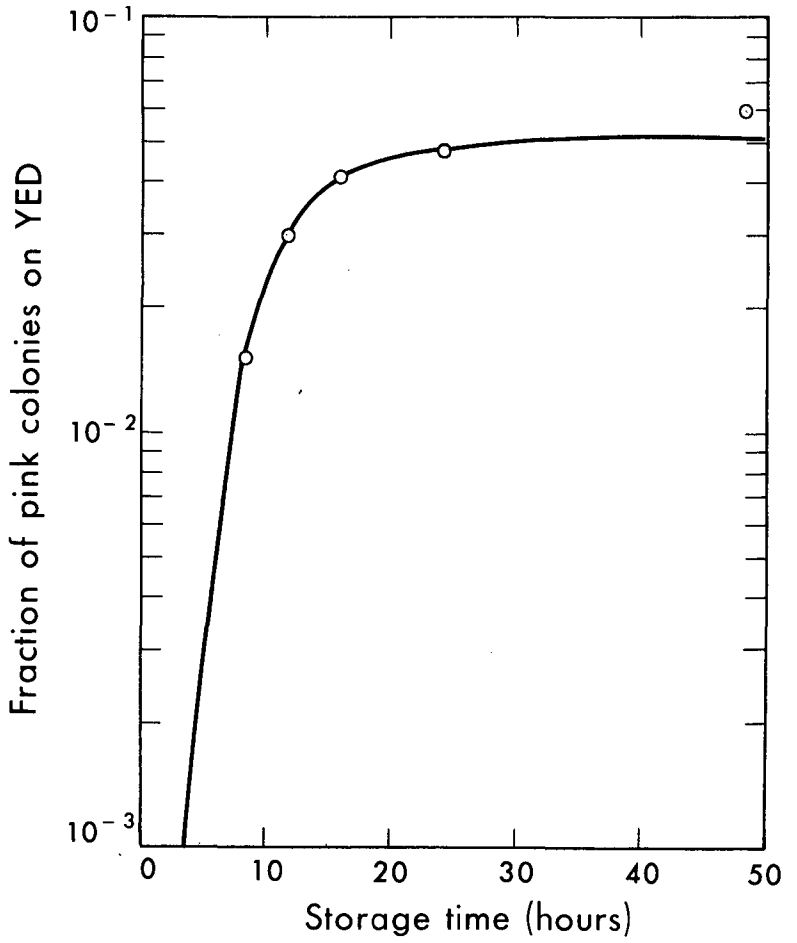
Fig. 25. Fraction of diploid -ar revertants of X841 after various periods in 0.3% potassium acetate in M/15 phosphate buffer (pH 6.0).



MU-35529

Fig. 26. Fraction of red colonies of YED media after various periods in 0.3% potassium acetate in M/15 phosphate buffer (pH 6.0). The solid curve has the same shape as the curve representing the fraction of haploid -ar revertants.

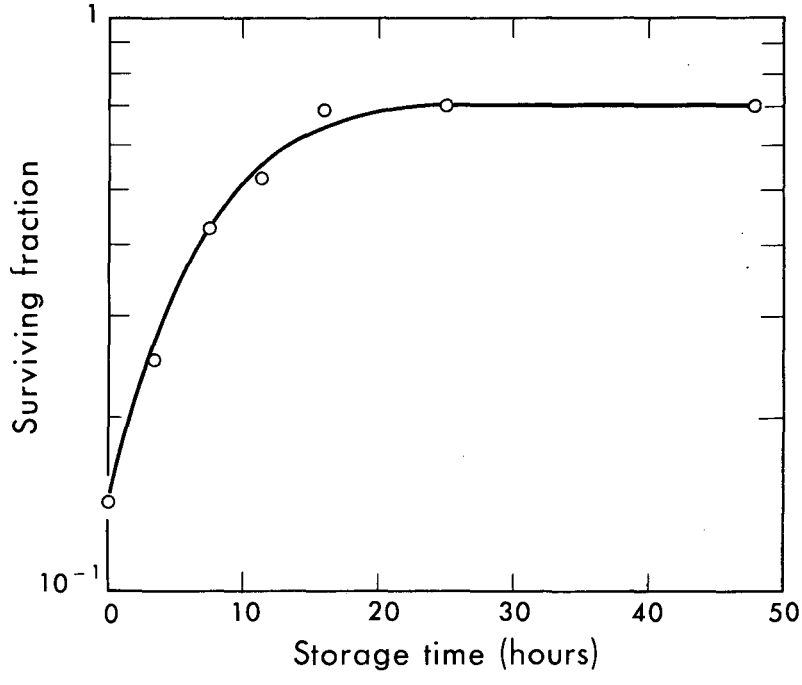


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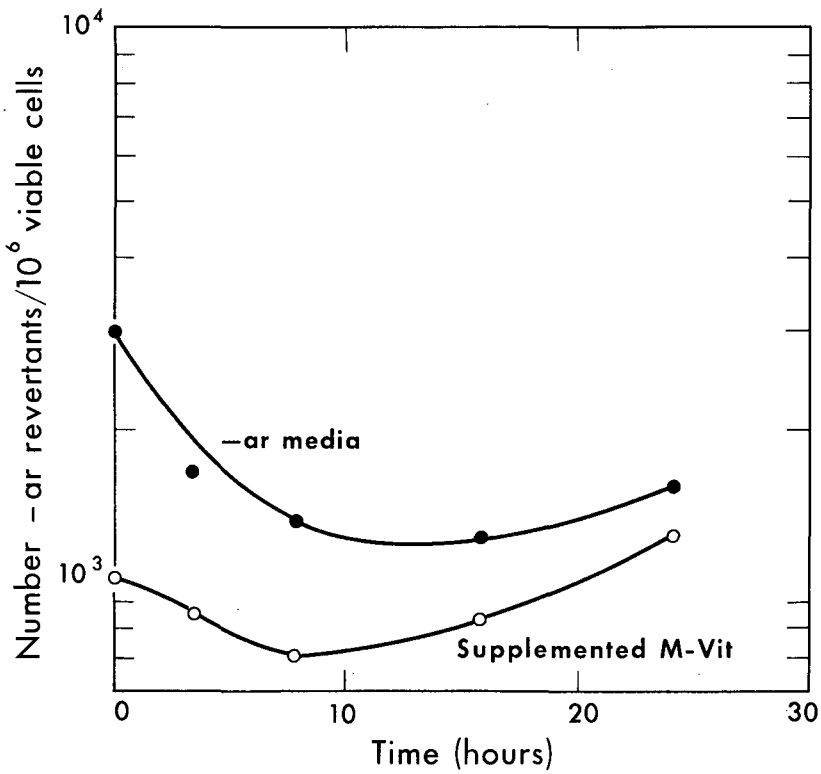
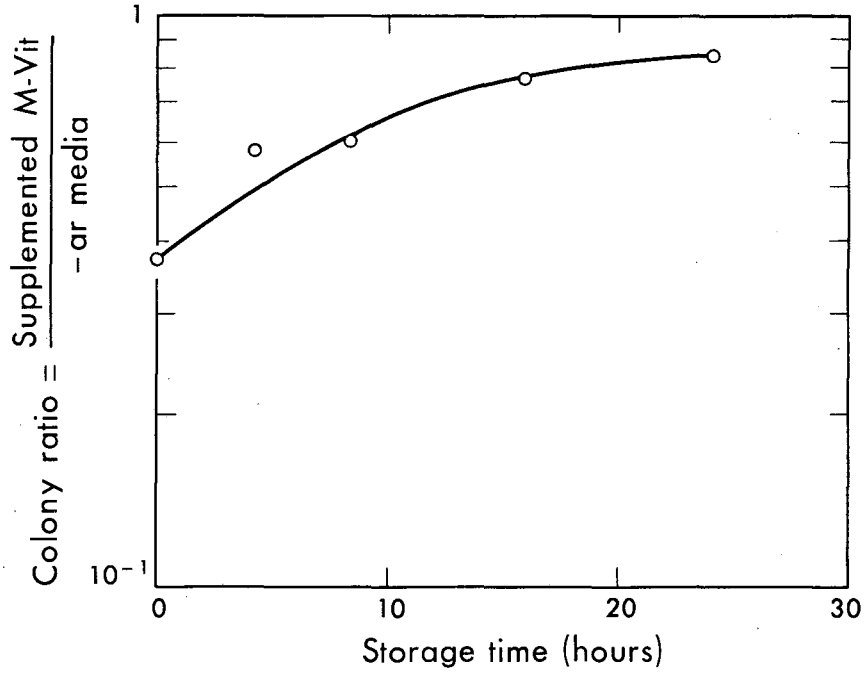
Fig. 27. Fraction of survivors after various periods in 0.3% potassium acetate in M/15 phosphate buffer (pH 6.0) following 75 krads from 50-kV x rays.



MU-35531

Fig. 28. Allelic recombination frequencies of irradiated (75 krad, 50-kV x rays) X841 cells, following postirradiation storage for various periods in 0.3% potassium acetate in M/15 phosphate buffer (pH 6.0).



MU-35532

Fig. 29. Ratio of revertant frequencies of irradiated cells of X841 determined by plating on supplemented M-VIT and -ar media at various times after storage at 30°C.

colonies (0-hour, -ar media) could now grow on supplemented M-VIT and only 10% of the colonies had requirements that could be satisfied by any of the five amino acids which constitute the difference between the two media. That is, by the time the cells have produced visible colonies, changes in the growth requirements had taken place and the additional amino acids were no longer required. The storage recovery treatment also reduces the dependence of some of the cells upon these additional nutrients.

After the first 8 to 10 hours, the frequency of revertant colonies begins to increase. This increase is presumably due to the type of allelic recombination that has been associated with DNA replication (77), since the recombination occurs prior to chromsome pairing in sporulation, as evidenced by the absence of haploid colonies. The low percentage of asci observed in the suspension indicated that the degree of sporulation is small. Miller (80) has recently reported that the ability to divide meiotically is more sensitive to ionizing radiation than the ability to divide mitotically. This seems to be borne out by the results of this experiment.

The above data suggest that storage recovery is not due to sporulation and that recovery occurs before DNA replication, that chromosome regions associated with induced allelic recombination are repaired, and that x-ray-induced allelic recombination has nutrient cofactors.

F. Mean Rate of Colony Formation

One of the effects of ionizing radiations is an inhibition of cell division, either temporary (division delay) or permanent (reproductive death). Division delay generally affects the first few divisions only (8). If the genetic damage is a dominant lethal lesion, the colony-forming ability will be inhibited in the first divisions or at least within a few cell divisions (81). When comparing irradiated haploid and diploid yeast after incubation on a nutrient medium, one observes that in the haploids, the microcolonies are limited to (a) normal colonies, (b) single cells, or (c) pairs of enlarged cells. When plating irradiated diploids,

one also observes chains of giant cells, apparently doomed to die, and quite often on these chains of cell of normal appearance arises from which a normal colony develops (12, 13, 82, 83).

In diploid cells, recessive lethal damage may be of such a nature that the genetic material is rearranged so that the progeny of such cells, for many generations, divide more slowly than a normal diploid (83, 84). When the progeny of a diploid survivor of ionizing radiation is followed through several generations during the postirradiation period, it is observed that not all the cells produced are able to divide again (81). In diploid and higher-ploidy cells, the presence of genetic material in two or more sets allows postirradiation rearrangements in the course of subsequent cell divisions and the appearance of cells with a normal rate of dividion. This is illustrated in Fig. 30.

Korogodin (85) has correlated the "mean rate of colony formation" with the radiation dose. The determination of this quantity is based upon a comparison of the number of visible colonies after 48 and (again) after 96 hours of incubation (i.e., $E = N_{48}/N_{96}$). This mean rate of colony formation has been determined for both the immediate and delayed platings following He^4-ion irradiation and is shown in Fig. 31. The 48-hour storage period resulted in rates of colony formation which can be compared with the rates of colony formation for immediate plating by the use of the DMF = 2.0.

The mean rate of colony formation is affected primarily by the amount of division delay, by the division rate, and by local environmental conditions surrounding the cell or colony. In order to determine which factors were being modified by the storage period, an analysis of the growth rate of the individual yeast colonies was performed. Through the kind assistance of Professor Donald Glaser, a specially designed incubator and associated photographic equipment were made available for the following experiment. This incubator has a revolving stage which accommodates 30 petri dishes, which are automatically positioned to be photographed one at a time by means of a 70 mm camera. This cycle may be repeated every 30 minutes or at longer intervals as desired. Colony image size was measured from the 70 mm

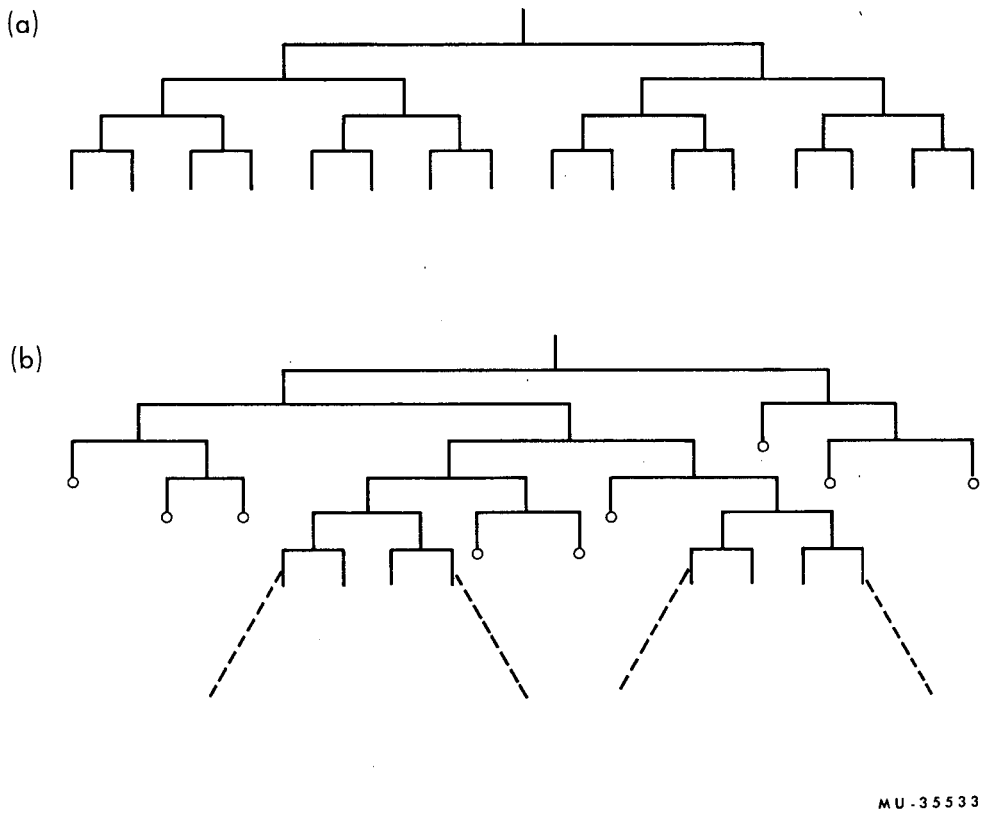


Fig. 30. Postirradiation division of diploid yeast. (a) Nonirradiated. (b) Irradiated. Open circles indicate cells which have stopped dividing. From Tobias (reference 81).

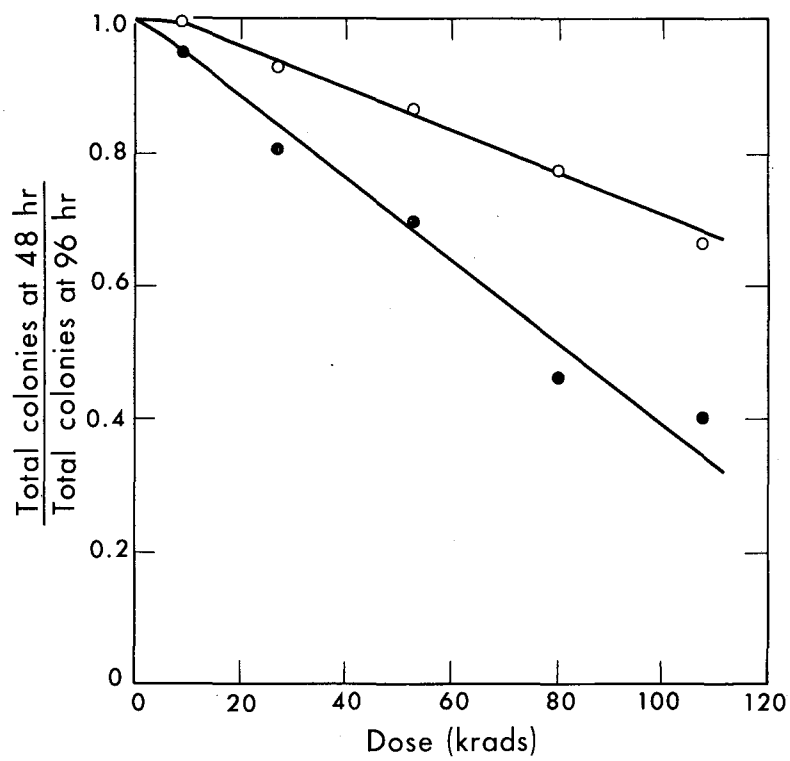


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Fig. 31. Mean rate of colony formation as determined by method of Korogodin (84) following He^4 ion irradiation. Delayed plating was 48 hours after immediate plating.

film, aided by a microscope projection comparator. Coordinates of the end points of two mutually perpendicular colony diameters were punched semiautomatically on IBM cards. These data from the time-lapse photographs of the 30 petri dishes were analyzed by an IBM 7094 computer and the output data were plotted by a CalComp Plotter. Figure 32 shows a redrawn graph of typical data obtained from the computer. The data are from six colonies on one petri dish. Colonies 1 and 2 were on an uncrowded section of the plate, while colonies 3 through 6 were grouped together. The average nearest-neighbor distance, measured between the centers of these four colonies, was 6.5 mm. A line drawing of this dish with the individual colonies identified is reproduced in Fig. 33. The effect of depletion of nutrients within the medium is not observed until these colonies are more than 3 mm in diameter. Data reported here were obtained for colonies which were separated from their nearest neighbor by at least 6.5 mm. Growth curves from these colonies are characterized by a portion in which the colony diameter increases linearly with time. This linear region is associated with colony diameters between 1 and 4 mm on YED plates used for this experiment. Colonies from irradiated cells fell into two categories. The first group had growth rates, during the linear portion of growth, which were indistinguishable from that of the unirradiated controls, but the time required to reach the linear portion of the curve was longer. This lag in growth is diagrammatically illustrated in Fig. 34. The second group of colonies was represented by growth curves which had much lower slopes (i.e., slower growth rates) and colony diameters which remained very small. This group was identified as consisting solely of petite mutants, identified as such by their white color and inability to grow on PET medium. These colonies were not further analyzed. The time required for the remaining colonies to attain a colony diameter of 2.5 mm was determined for both the immediate and delayed platings following x irradiation. The number of hours the colonies from irradiated cells lagged behind the controls for each plating is shown in Fig. 35. The two curves are comparable if one uses a DMF = 3.0. These results indicate that by the time that a colony has

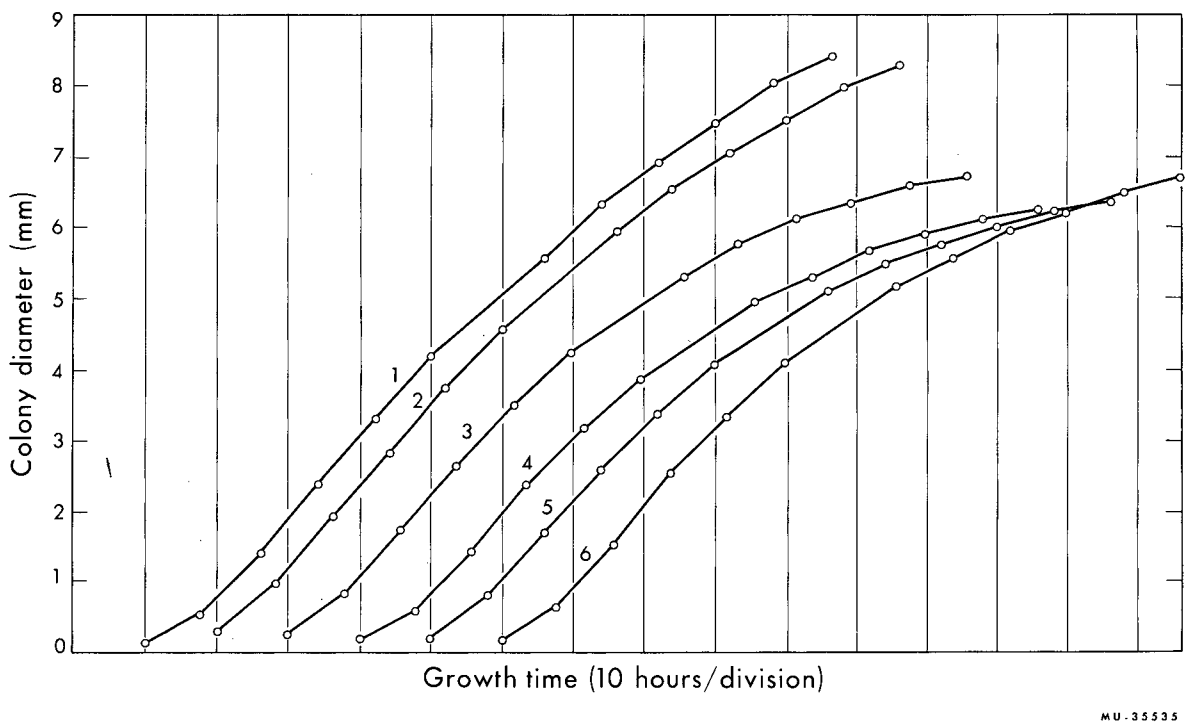

Fig. 32. Yeast colony growth curves. Colonies 3 through 6 demonstrate influence of colony crowding on colony diameter. Average nearest neighbor distance for colonies 3 through 6 was 6.5 mm.

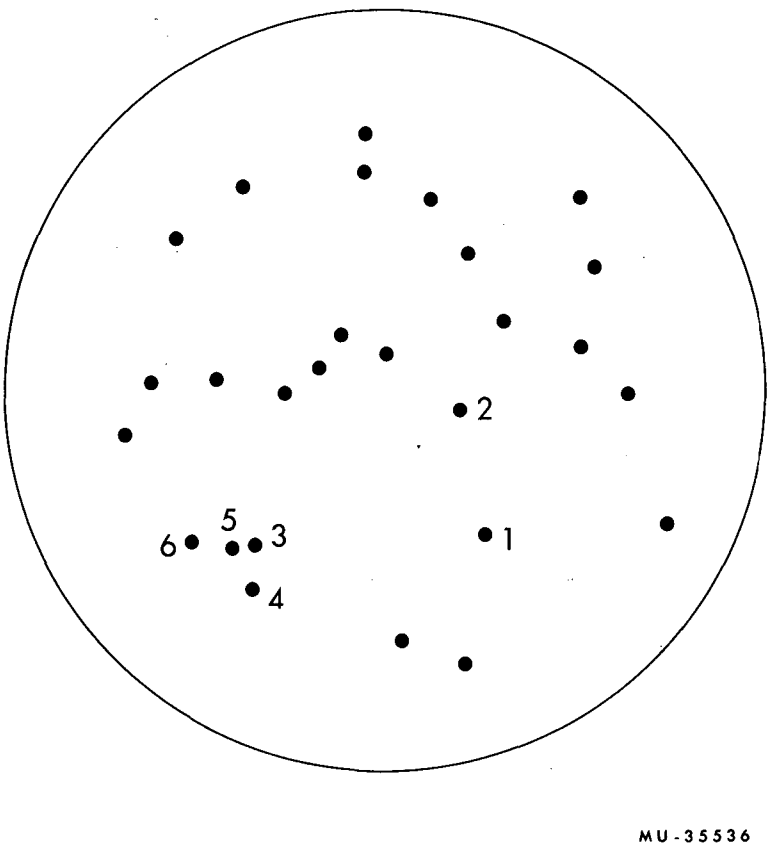

Fig. 33. Line drawing of petri dish showing location of colonies 1 through 6 of Fig. 32.

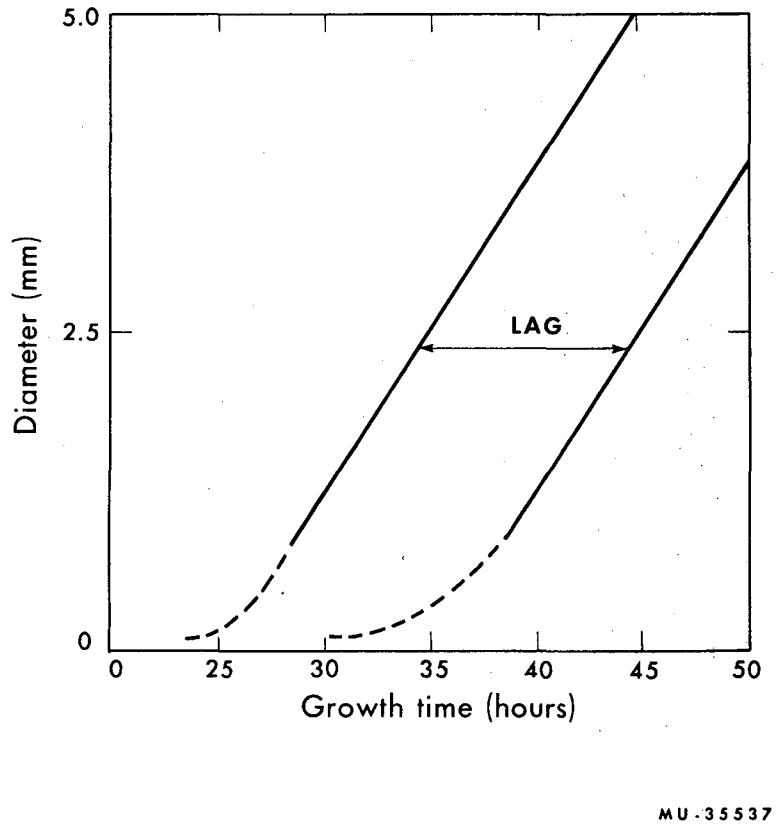

Fig. 34. Diagrammatic drawing of typical growth curves for unirradiated and irradiated normal yeast cells.

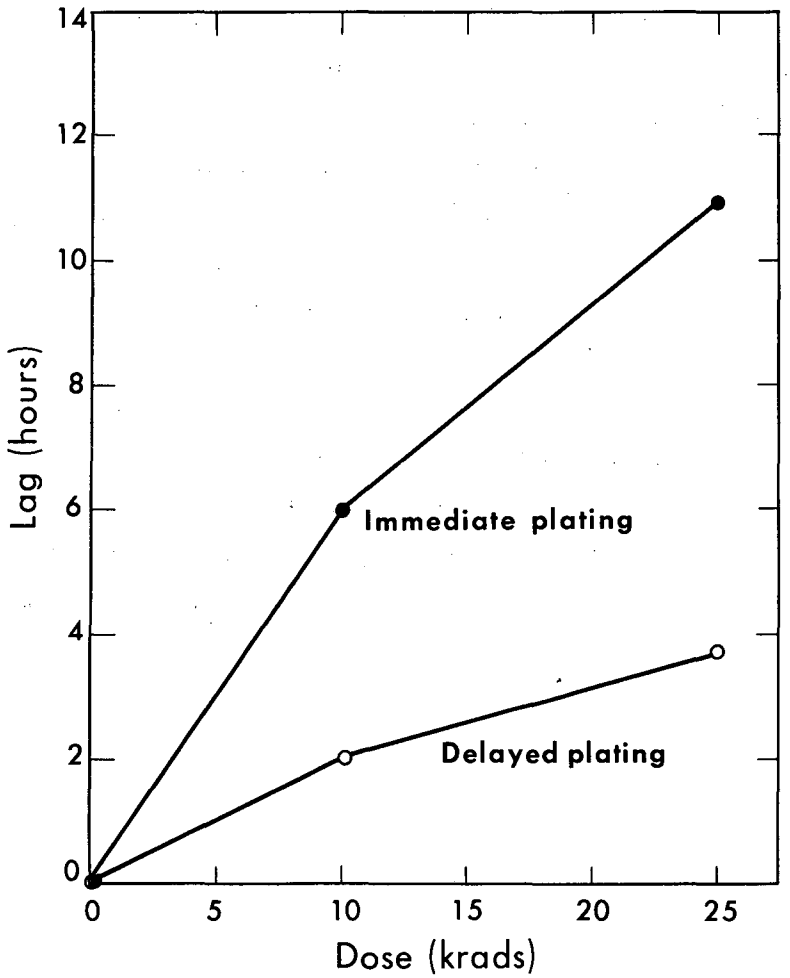

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Fig. 35. Lag time for irradiated colonies following 50-kV x irradiation. Delayed plating 24 hours after immediate plating.

attained a diameter of 1 mm, if not sooner, normal dividing cells greatly outnumber any more slowly dividing cells, so that measured growth rates of these colonies are the same as for colonies from unirradiated cells. The reduction in the time lag, then, occurs because the division delay of the first few division cycles is reduced, or because normally dividing cells appear in an earlier generation following delayed plating than following immediate plating. The lag time represents the additional time required by an irradiated cell or the microcolony from the irradiated cell to produce a cell with a normal growth rate.

Among the irradiated cells which are not capable of forming a visible colony, it has been reported (24) that the average number of cells in the nonviable microcolony is larger following delayed plating than following immediate plating. This indicates that even among the nonsurvivors there has been a lessening of the radiation damage.

IV. DISCUSSION

The experiments reported here have been hopefully performed with the objective of obtaining additional information on the nature of the postirradiation storage recovery mechanism. It had been shown previously that the recovery mechanism could "repair" lesions associated with oxygen-dependent and oxygen-independent damage with equal efficiency. It was also known that recovery was possible following α-particle irradiation. It has now been shown, in this paper, that diploid yeasts are also capable of recovering from densely ionizing heavy-ion irradiation ($dE/dx = 4920$ MeV-cm^2/g) and that recovery, within the experimental error, is the same as observed following x radiations. Three possible mechanisms have been suggested as possible recovery mechanisms. With genetically marked yeast strains, I have attempted to distinguish between the proposed mechanisms. Although there are a great many similarities between the conditions used for recovery and those for sporulation, the latter is not a prime recovery mechanism, as evidenced by recovery of the homozygous mating-type strain, XJ4. This does not rule out the possibility that recovery and sporulation may be related, since they might share common pathways, and that the step which prevents the homozygous mating-type strains from sporulating occurs after the possible sharing of pathways. Allelic recombination frequencies based on survivors are decreasing during the time of the maximum storage-recovery rate, indicating that lesions associated with recombination are being repaired. This is counter to the observation that would be expected if recombination were an important recovery mechanism. Reunion of broken segments or repair of damaged segments, as opposed to recombination, is the more likely explanation. Allelic recombination which has been associated with DNA synthesis occurs after the recovery rate is decreasing, suggesting that DNA synthesis does not start until recovery is complete, or nearly complete. This leaves direct enzymatic repair as the most likely of the proposed recovery mechanisms. If this is the case, then

the observation that the induced allelic recombination cofactors disappear might possibly be explained by the temporary inactivation of a wild-type gene at a locus which is heterozygous. During the storage the damaged gene is enzymatically repaired, and if it is not repaired during this time, it is still possible to repair the gene after the cell has been placed on the nutrient media.

V. CONCLUSIONS

Recovery of diploid yeast cells has been observed following both lightly ionizing ($dE/dx = 20$ MeV-cm^2/g) x irradiations and densely ionizing heavy-particle irradiations ($dE/dx = 4290$ MeV-cm^2/g), and within the experimental error there is no change in efficiency of the repair mechanism with changes of the LET. Storage recovery has been shown to be additive to and independent of glycerol protection. The ability to sporulate is not a required condition for a recovery mechanism to be functional, nor is the heterozygosity of the mating-type allele. Postirradiation storage of normal diploids leads to (a) increased viability, (b) reduction of allelic recombination frequencies, (c) reduction in dependence of revertant colonies on the more complete of two omission media, (d) reduction in the time to form a visible colony.

The failure of any demonstration of a recovery mechanism in haploid yeast that operates in cells damaged by ionizing radiations suggests that the recovery mechanism, as studied, might be affecting primarily the dominant lethal, but not the recessive lethal damage. This would account for the failure of recovery experiments with haploids, since haploids are inactivated almost exclusively by recessive lethal damage (6). The hypothesis that recovery is primarily through repair of dominant lethal damage might be tested by measuring zygote viability following irradiation and storage of one haploid parent. If an increased viability is observed with increased storage time, this should indicate that there is a recovery mechanism in haploid yeast which can repair dominant lethal damage in the haploid cell.

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