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The Shape of Dose–Survival Curves for Mammalian Cells and Repair of Potentially Lethal Damage Analyzed by Hypertonic Treatment

W. POHLIT AND I. R. HEYDER

Gesellschaft für Strahlen- und Umweltforschung mbH Abteilung für Biophysikalische Strahlenforschung, Paul-Ehrlich Strasse 20, D6000 Frankfurt am Main, Germany

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During the usual procedure of testing cell survival by colony-forming ability, repair of potentially lethal damage (PLD) takes place. By incubating the cells in hypertonic suspension a certain part of this repair can be inhibited, leading to an exponential dose–survival curve as expected from the Poisson distribution of lethal events in the cells. If such a hypertonic treatment is performed after increasing intervals following irradiation with X rays, curves with increasing shoulder length are obtained. Quantitative analysis of the kinetics of this repair shows that PLD is repaired for about 1 hr after irradiation by a saturated repair system which eliminates about one lesion per 15 min per cell independent of the applied absorbed dose. PLD not eliminated by this "fast" system is repaired by an unsaturated system with a time constant of several hours. Repair of PLD after X irradiation proceeds quantitatively in this way in plateau-phase cells suspended in a conditioned medium, which seems optimal for such repair. If these cells are suspended after irradiation in normal nutrient medium a certain fraction of the PLD is transformed into irreparable damage. The final survival after repair in nutrient medium is then identical with that obtained by the usual measurement of colony-forming ability on nutrient agar. This indicates that the shoulder in dose–survival curves for plateau-phase cells is partly due to repair of PLD and partly due to manifestation of this damage during repair time.

INTRODUCTION

It is well established that mammalian cells are able to repair potentially lethal radiation damage (PLD) both in the growth phase (*1–4*) and in the plateau phase (*5–13*). Operationally, repair of PLD is defined as the increase of survival during an interval after irradiation before the viability of the cells is tested by their colony-forming ability. In both cases—with and without repair of PLD—dose–survival curves of the shouldered type are usually obtained. The radiation sensitivity of the cells can be increased by treatment with hypertonic buffer solutions (*14–22*), and it has been shown that this leads to an increased number of observable potentially lethal events in the irradiated cells (*23*).

Quite different conclusions have been derived from these facts, especially with respect to their influence on the shape of the dose–survival curve (*22*, *24*). Different

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types of reactions have been assumed (*22*) for repair of PLD and for sublethal damage (SLD), and the accumulation of SLD was assumed to be responsible for the shoulder in the cell survival curve. This was mainly derived from the number of lesions produced in the DNA, such as single- and double-strand breaks, and their time constants of repair compared with those for cell survival. Another study (*20*), however, shows about the same influence of anisotonic treatment on the inhibition of repair of PLD and SLD. Repair of SLD was questioned (*24*) as a "misleading association with Elkind recovery," and an exhaustion of the repair capacity with increasing absorbed dose was assumed for a quantitative description of the shape of survival curves as proposed earlier (*25*).

The basic biochemical mechanism leading to the shoulder in dose–survival curves has an important impact on several aspects of the practical use of ionizing radiation in tumor therapy and on expectations of radiation damage at low absorbed doses in radiation protection. Therefore the purpose of this investigation was to test the above-mentioned assumptions of quantitative analysis of the repair of PLD in a homogeneous population of mammalian cells.

Cells in the plateau phase of growth were chosen to obtain a population with suitable homogeneity and to allow for long repair times under fairly constant physiological conditions. The cell line used here is able to proliferate in suspension (*11*) and as a monolayer (*13*). These cells will also grow as an ascites in the abdomen of mice and produce solid tumors in the leg muscle. The data presented here on repair of PLD in cell suspension can be tested, therefore, under a variety of biological environmental conditions.

MATERIALS AND METHODS

Cultivation of the Cells

The method of cultivation of Ehrlich ascites tumor cells and the composition of the nutrient medium used in these experiments have been described already in detail (*11*); only some general facts will be discussed here. The main features of the nutrient medium [F-Med., all components are given in (*11*)] are: (1) A high buffer capacity (36 mmole/liter $NaHCO_3$ in an atmosphere of 6% CO_2 kept the culture at pH above 7 throughout exponential growth and in the plateau phase. (2) An organic buffer of 20 mmole/liter Hepes was added to avoid pH changes during manipulation of the cells outside the incubator. (3) A glucose concentration of 27 mmole/liter was used to assure sufficient viability of the cells in the plateau phase for more than 2 days.

From exponentially growing cells in suspension, subcultures with a cell concentration of $N_0 = 10^5$ cells/ml were prepared in fresh nutrient medium and were incubated for 72 hr at 37°C. The cells then entered the plateau phase at a concentration of 2.2×10^6 cells/ml and were used for irradiations. Cell concentrations were measured by an electronic cell counter (Fa-Coulter).

Preparation of Hypertonic Solutions

To make a comparison with recently published results (*22*) a phosphate buffer was used in several experiments with different osmotic pressures produced by addition of sodium chloride.

A medium was prepared from cell suspensions grown to the plateau phase as described above. Most of the cells were removed from the medium by centrifugation and the rest by filtration. Due to the very low cell concentration after centrifugation, contamination of the medium from cells destroyed on the filter was negligible. The molar concentration responsible for the isotonic osmotic pressure p_{iso} of this conditioned medium (C-Med) was $C = 270$ mmole/liter.

The osmotic pressure of the different hypertonic solutions, p_{os}, was adjusted by adding suitable amounts of sodium chloride. The pH value of these solutions remained unchanged at pH = 7.4 due to Hepes buffer in the nutrient medium. Throughout the paper reactions are related to the relative osmotic pressure p_{os}/p_{iso}.

Measurement of ATP Content

The ATP-consuming and light-emitting reaction of luciferin with the enzyme luciferase was used to determine the ATP content of the cells under different physiological conditions. Volumes of $V = 0.01$ ml of cell suspension with a concentration of at least $C_N = 4 \times 10^4$ cells/ml were mixed for 30 sec with nucleotide-releasing agent for somatic cells (NRS, Abimed) at room temperature (25°C). Then 0.1 ml of luciferase–luciferin solution in Hepes buffer was added, and exactly 15 sec later the measurement of emitted photons was started. The number of photons was integrated over 10 sec; this integration was repeated for another 10 sec. The count rate was about 800,000 counts in 10 sec. A fixed amount of ATP (200 pg in 0.01 ml) was added exactly 15 sec later, and the increased photon emission of about 100,000 counts/10 sec was integrated again over two subsequent 10-sec periods. In this way the counter was calibrated for each measurement with an overall uncertainty of less than 2%. Statistical fluctuations in the count (integral) and the background (about 400 in 10 sec) can be neglected. An uncertainty of about 5% resulted when a low number of cells per sample was used (at minimum about 400). The ATP content of the cells was determined by extrapolating the two first measurements with the cell suspension back to the time of the luciferin–luciferase addition (uncertainty less than 1%). For exponentially growing cells the ATP content was $C_{ATP} = 5$ pg/cell. For the measurement of the reduced cellular ATP content in plateau-phase cells or in hypertonic solutions, higher cell concentrations were chosen to reduce the statistical uncertainties.

Measurement of Cell Volume

The volume of cells at different osmotic pressures was measured by a commercial cell counter (Telefunken-Coulter), in which the cell suspension is injected as a thin hydrodynamically focused stream through a diaphragm. Cell volume is measured from changes of conductivity between two electrodes by pulse height analysis with a multichannel analyzer. Linearity of the pulse height–volume relation was checked with latex particles of known diameter. The conductivity of the solutions with different osmotic pressures was different due to the varying sodium chloride content. Therefore the pulse height analyzer was calibrated for each solution using mulberry

spores with a mean diameter of 12.3 μm which could be measured with a precision of about ± 0.1 μm.

Irradiation

Cells were irradiated at room temperature (25°C) in suspensions with cell concentrations of 2×10^6 or 2×10^5 cells/ml, which were stirred continuously during irradiation. In this way uniform distribution of the cells in solution and sufficient supply of oxygen are guaranteed. X rays were used with a tube voltage of 140 kV and with a 0.7-mm aluminum filter. The effective photon energy was then about 20 keV with respect to the photon attenuation coefficient of water. The absorbed dose was monitored during irradiation by a transmission ionization chamber which had been calibrated by an energy-independent chemical dosimeter (*26*). The absorbed dose rate was about 10 Gy/min, resulting in irradiation times of less than 1 min.

Cells were diluted after irradiation into fresh nutrient medium and were plated on nutrient agar as described (*11*). The plating efficiency of unirradiated cells was between 0.8 and 0.9, and colonies were counted after 2 weeks of incubation at 37°C. Usually for each experimental point four dishes, each with 100 to 200 colonies, were counted, resulting in statistical uncertainties of about 5% or less. Colony-forming ability or cell survival is expressed by the quotient of the counted macrocolonies after irradiation N_A and the unirradiated control N_{A0} taking account of the dilution factors used.

RESULTS AND DISCUSSION

Effect of Hypertonic Treatment on Cell Functions

Since hypertonic treatment was to be used here for analyzing radiation reactions we felt it important to evaluate the influence of such a treatment on the normal physiological functions of the cells used.

Proliferation in nutrient medium and ATP content. The growth of cells was measured in nutrient media with the same basic composition (F-Med) but different osmotic pressures (see above). As can be seen from the results in Fig. 1 an osmotic pressure p_{os} of 1.8 times the isotonic value p_{iso} results in only small changes in cell proliferation. At twice the isotonic concentration cells are able to proliferate after an initial lag time of about 12 hr at about half the normal cell doubling time. At higher osmotic pressure cell growth is retarded, possibly caused by cell loss due to a lack of energy production. As can be seen from Fig. 2 a drastic reduction in ATP content occurs in cells kept at $p_{os}/p_{iso} = 2.2$ correlated with a large cell loss from the population. In isotonic solution the ATP content remains constant at about $C_{ATP} = 5$ pg/cell during the exponential phase of growth. When the cells enter the plateau phase at about 96 hr the ATP content drops to about $C_{ATP} = 2.5$ pg/cell and then remains constant for at least 24 hr, during which the repair experiments with plateau-phase cells have been done.

ATP content of plateau-phase cells in conditioned medium. Cells grown into the plateau phase as described were diluted into conditioned media with different

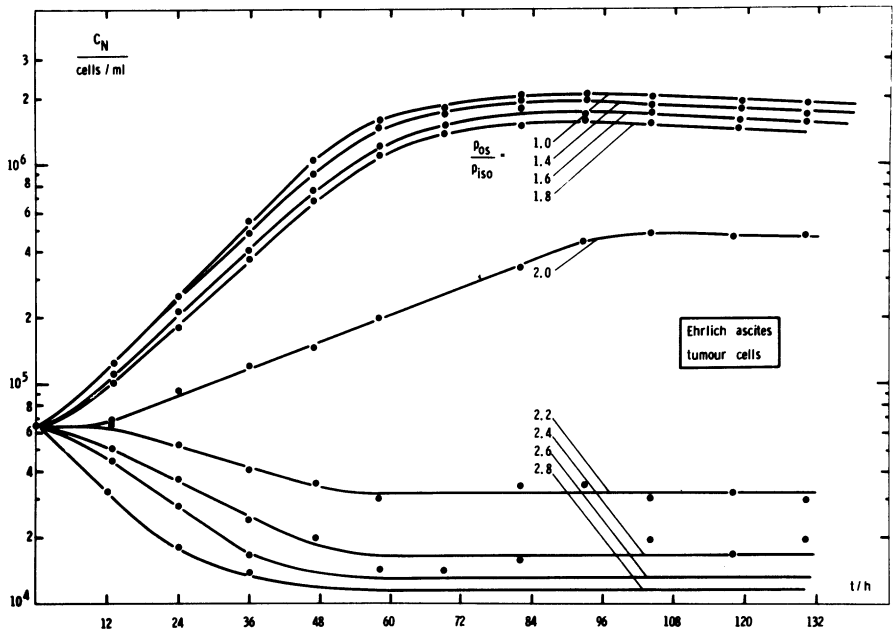


FIG. 1. Cell growth at 37°C in nutrient medium with different relative osmotic pressures p_{os}/p_{iso} (p_{os} = osmotic pressure; p_{iso} = isotonic osmotic pressure corresponding to concentration of C = 270 mmole/liter). Cell concentration C_N is plotted as a function of time t.

osmotic pressures and were kept at 37°C. The ATP content of these cells is shown in Fig. 3 as a function of time. In isotonic solutions the ATP content first increases and then returns to its initial value after about 10 hr. At osmotic pressures higher than about $p_{os}/p_{iso} = 2.5$ the ATP is reduced from the outset and reaches concentrations of less than 1/10th the initial concentration after more than 5 hr. The cells then start to die, as has been measured by determination of colony-forming ability of these cells. In the irradiation and repair experiments which will be described later a relative osmotic pressure of $p_{os}/p_{iso} = 1.9$ was applied for $t = 80$ min. As can be derived from Fig. 3 no influence from inhibition of the energy metabolism can be expected.

The same changes in ATP content as in Fig. 3 were observed (within experimental uncertainties of about 5%) for cells irradiated with an absorbed dose of $D = 10$ Gy, indicating that energy production in these cells is not influenced by irradiation within the dose range used here.

Change in cell volume in hypertonic solutions. If a cell is incubated in a hypertonic solution with relative osmotic pressure p_{os}/p_{iso} its volume under isotonic condition $V_{c,iso}$ is reduced to $V_{c,os}$. If a partial volume V_n of the cell is incompressible with respect to an osmotic difference, then

$$V_{c,os} = (p_{iso}/p_{os}) \cdot V_{p,iso} + V_n, \tag{1}$$

where $V_{p,iso}$ is the partial cell volume under isotonic condition which is compressible

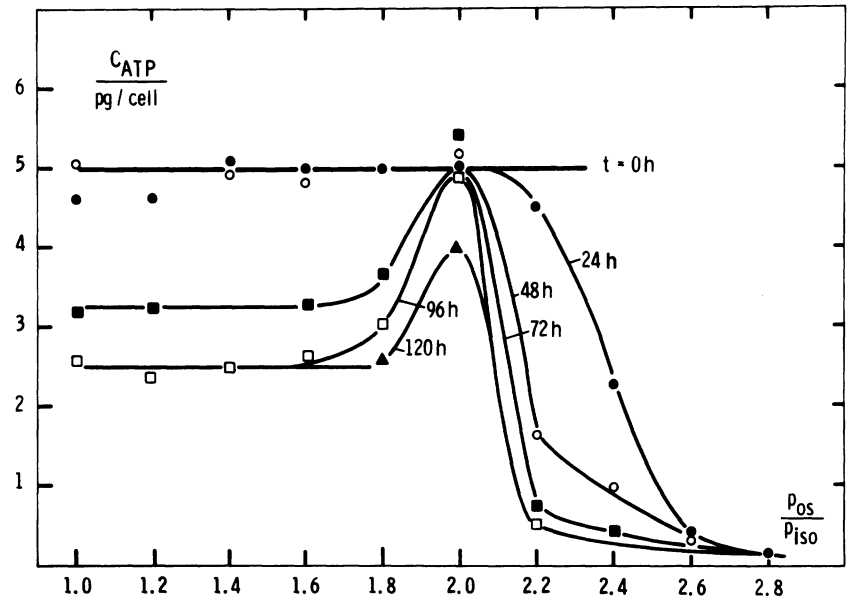


FIG. 2. ATP content of Ehrlich ascites cells after different times t of growth in nutrient medium (see Fig. 1) at various relative osmotic pressures. The horizontal line indicates the value of $C_{ATP} = 5.0 \pm 0.2$ pg/cell measured at $t = 0$ for all osmotic pressures. (Uncertainty of C_{ATP} measurements: $\pm 5\%$.)

with respect to an osmotic difference. This equation corresponds to the well known Boyle-Van't Hoff equation. From measurement of $V_{c,os}$ at different p_{os}/p_{iso} the incompressible part of the cell was determined to be about $V_n = 100\ \mu m^3$ ($\pm 20\ \mu m^3$), which is less than 1/10th the total cell volume $V_{c,iso} = 1400\ \mu m^3$ ($\pm 100\ \mu m^3$) of plateau-phase Ehrlich ascites cells. The nucleus of the cell therefore cannot be considered to be completely incompressible, and consequently changes in its structure are understandable in hypertonic solution as has been observed with other methods (*20*). These changes in structure may then have an influence on the radiation sensitivity and on repair processes in irradiated cells as described below.

Effects of Hypertonic Treatment on Radiation Reactions

Influence of hypertonic treatment time on cell survival after irradiation. Plateau-phase cells were incubated immediately after irradiation in phosphate buffer to enable comparison with results obtained with Chinese hamster cells (*23*). A relative osmotic difference $p_{os}/p_{iso} = 1.9$ was chosen to omit influences on energy metabolism during intervals of several hours (see Figs. 2 and 3). Cell survival N_A/N_{A0} (N_A = number of cells with colony-forming ability after irradiation; $N_{A0} = N_A$ for $D = 0$) after irradiation with $D = 4$ Gy decreases with treatment time as shown in Fig. 4. The experiments with Chinese hamster cells were performed with 20 min treatment time because of a break in this curve found at about $t_{os} = 20$ min (*23*). For comparison with these data Ehrlich ascites cells were also treated for $t_{os} = 20$ min in hypertonic phosphate buffer (see Figs. 5 and 6).

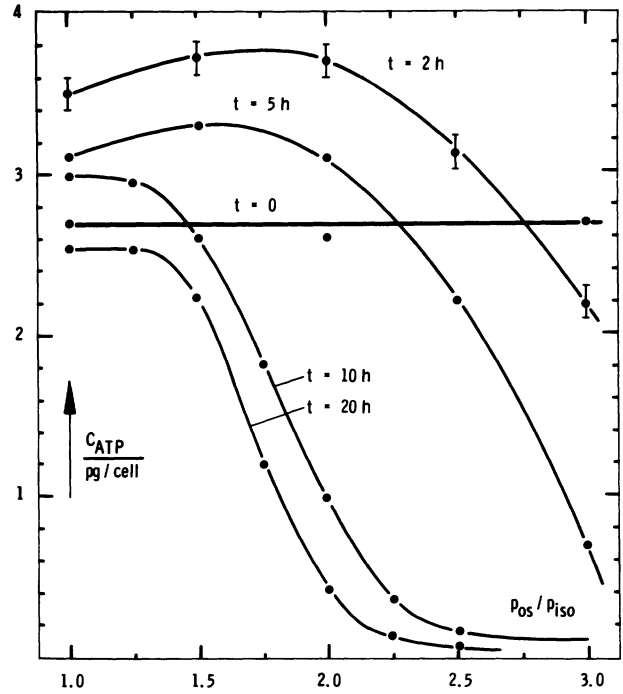


FIG. 3. ATP content of plateau-phase Ehrlich ascites cells after different times t of storage at 37°C in conditioned medium with different osmotic pressures. (Uncertainty for all C_{ATP} measurements is ±5% as indicated for t = 2 hr.)

Cells incubated after irradiation in conditioned medium (C-Med) with p_{os}/p_{iso} = 1.9 showed approximately the same initial decrease in survival but a more emphasized plateau for treatment times between t_{os} = 40 and 80 min. This plateau indicates that a certain type of repair process is inhibited with a hypertonic treatment of p_{os}/p_{iso} = 1.9 and t_{os} in the plateau region. With longer treatment times an additional effect seems to take place which further reduces the survival. The influence on unirradiated control cells after these times could also no longer be neglected. Therefore the experiments with hypertonic C-Med were done with a treatment time of t_{os} = 80 min.

Repair of potentially lethal damage in conditioned medium. Plateau-phase cells were irradiated in their own C-Med at room temperature (25°C) with absorbed doses up to 8 Gy. They were diluted immediately after irradiation into hypertonic phosphate-buffered saline at 37°C and left there for 20 min. After this treatment they were diluted further in fresh nutrient medium (F-Med) and plated on nutrient agar. If the cells were kept between irradiation and hypertonic treatment in C-Med at 37°C for a certain repair time, t_{rep}, then cell survival increased as shown in Fig. 5.

If the hypertonic treatment was performed for 80 min in hypertonic C-Med, curves were obtained similar to those with hypertonic buffer. If these curves for hypertonic C-Med were shifted on the time axis t_{rep} in Fig. 5 by about 15 min the

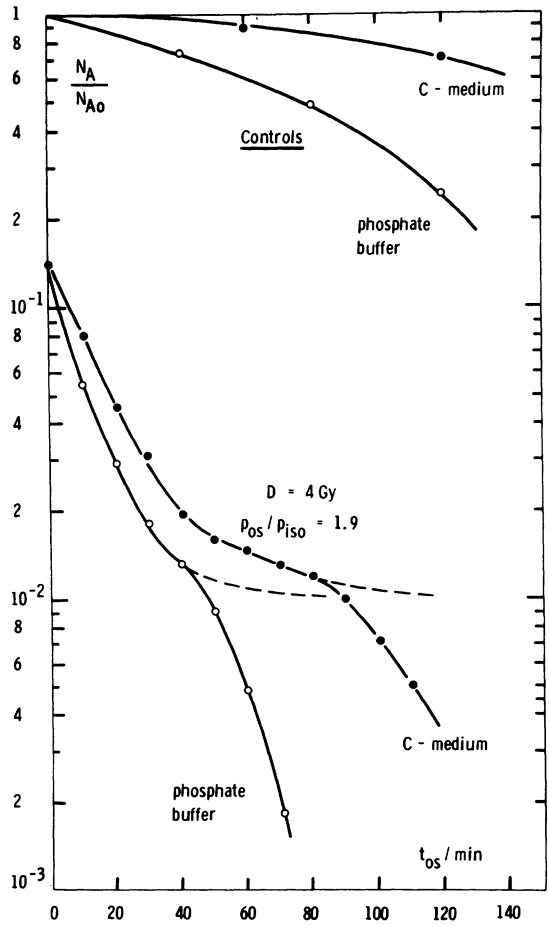


FIG. 4. Relative cell survival N_A/N_{A0} as a function of treatment time t_{os} in hypertonic phosphate buffer (O) and in hypertonic conditioned medium (●). The data for irradiated cells have been normalized by the controls at corresponding times.

two sets of repair curves agreed completely for all repair times within experimental uncertainties of about ±5%. Only the initial values of survival at $t_{rep} = 0$ for the hypertonic treatment with hypertonic C-Med were much lower as indicated in Fig. 5. (Other data with hypertonic C-Med are not shown in Fig. 5 for clarity.)

The agreement of the complete course of the repair curves obtained with the two different types of hypertonic treatment indicated that the biological repair mechanism measured in this way was largely independent of the particular conditions imposed by these two methods such as phosphate concentration. The differences at $t_{rep} = 0$ can be understood from the results shown in Fig. 4 for both treatments. At a dose of 4 Gy survival after 20 min of hypertonic buffer treatment is about two times higher than that after treatment in hypertonic C-Med. This indicates that insufficient treatment time (20 min) allows the cells to repair a certain part of potentially lethal damage on the nutrient agar corresponding to a repair time of about 15 min in C-Med, as will be discussed later.

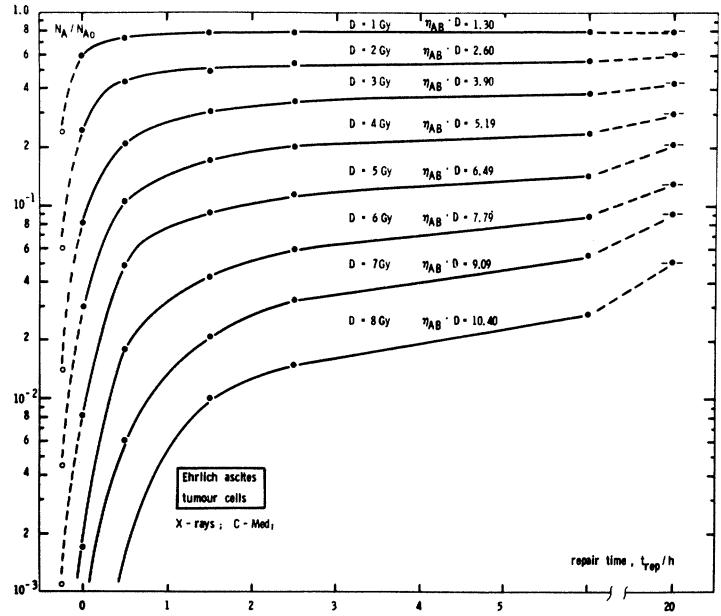


FIG. 5. Increase of cell survival N_A/N_{A0} as a function of repair time, t_{rep}, in conditioned medium at 37°C. Cells were treated after t_{rep} with hypertonic phosphate buffer (●) or hypertonic conditioned medium (○).

The data presented in Fig. 5 can be used also to draw dose–survival curves for different repair times as shown in Fig. 6. For treatment with hypertonic C-Med an exponential dose–survival curve is obtained. With increasing repair time first a shoulder is produced and later also the final slope of the dose–survival curve is decreased. The indicated repair time t_{rep} corresponds to the experimental data obtained with hypertonic C-Med. Therefore the curve shown for $t_{rep} = 0.25$ hr corresponds to $t_{rep} = 0$ for a treatment with hypertonic buffer. This result agrees with published data obtained with hypertonic phosphate buffer in Chinese hamster cells, where a dose–survival curve with a smaller shoulder and a steeper slope was also measured (*23*) compared with the dose–survival curve without hypertonic treatment. For comparison the dose–survival curve without hypertonic treatment (immediate plating, IP) is given in Fig. 6.

After long repair times ($t_{rep} = 20$ hr) no influence of the hypertonic treatment can be observed. The dose–survival curves are identical with those for delayed plating (DP).

As can be seen from the experimental data in Fig. 5 or 6 three types of cells may be distinguished after irradiation (see also Appendix II):

state A: cells with colony-forming ability;

state B: cells with reparable damage;

state C: cells with irreparable damage.

The relative number of cells in state A can be read from Fig. 5 or 6 as N_A/N_{A0}.

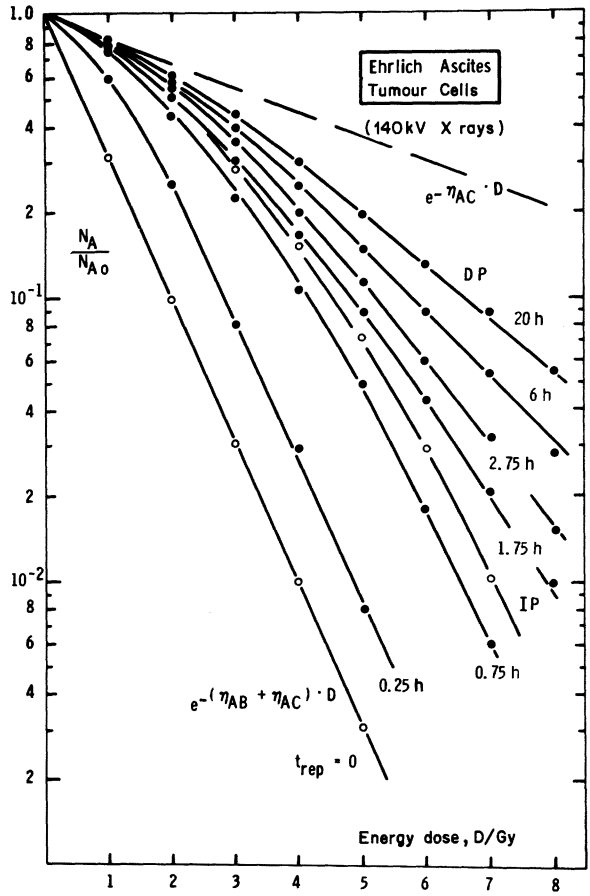


FIG. 6. Dose-survival curves for different repair times, t_{rep}, in conditioned medium at 37°C. Cells were treated after t_{rep} with hypertonic phosphate buffer (●) or hypertonic conditioned medium (O). Curve IP: immediate plating without repair and without hypertonic treatment; curve DP: delayed plating after t_{rep} = 20 hr without hypertonic treatment.

The relative number of cells in state C can be obtained from N_A/N_{A0} after a sufficient repair time ($t_{rep} \rightarrow \infty$):

$$N_c/N_{A0} = 1 - (N_A/N_{A0})_{t_{rep}} \rightarrow \infty \qquad (2)$$
$$= 1 - S_\infty$$

(where $N_A/N_{A0} = S$ and the index indicates the repair time).

Accordingly the relative number N_{B0} of cells in state B immediately after irradiation is

$$N_{B0}/N_{A0} = S_\infty - S_0 \qquad (3)$$

(where S_0 is the survival N_A/N_{A0} at $t_{rep} = 0$).

After a repair time some of the cells have left state B by repair; the relative

number is $S(t_{rep}) - S_0$. Therefore the number of cells in state B after repair, N_B, relative to this number at $t_{rep} = 0$ (N_{B0}) is given by

$$\frac{N_B}{N_{B0}} = 1 - \frac{S(t_{rep}) - S_0}{S_\infty - S_0}. \tag{4}$$

It is shown in Appendix II that reparable and irreparable radiation damage can be treated separately, and therefore Eq. (4) can be used for analyzing the repair of PLD by using the experimental survival data of Fig. 5 or 6. In Fig. 7 N_B/N_{B0} is drawn in a semilog plot as a function of repair time from the curves of Fig. 5 using additional experimental data, which for clarity are not shown in Fig. 5. As can be seen from Fig. 7 the repair, i.e., the decrease of cells in state B, does not proceed with a single time constant which would result in straight lines. In contrast a rather complicated dependence on repair time and absorbed dose was apparent.

As is shown in detail in Appendix I, repair of potentially lethal damage can be calculated starting from a Poisson distribution of these lesions in individual cells. Such calculated curves are drawn in Fig. 7 and show good agreement with the experimental results for the first 1 to 2 hr of repair. The course and the initial slope at different absorbed doses are explained in this way by a saturated repair system which eliminates, independent of absorbed dose, four potentially lethal lesions per hour in the individual cells (for details see Appendix I).

After longer repair times the experimental results in Fig. 7 indicate repair with a time constant of about 8 hr independent of the absorbed dose.

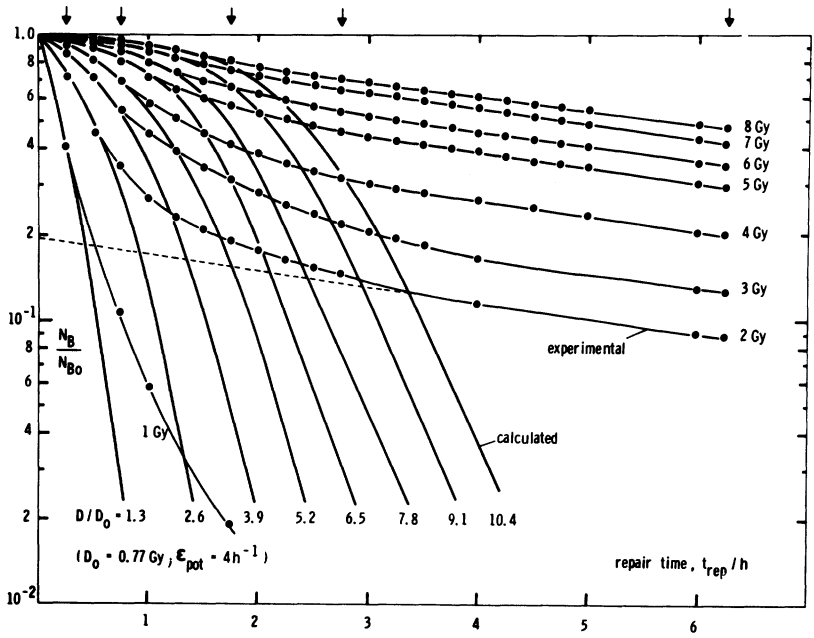


FIG. 7. Decrease of relative cell number in state B with PLD as a function of repair time in conditioned medium at 37°C (experimental data from Fig. 5; calculated curves from Fig. 12a).

For detailed analysis of such repair curves in Fig. 8a experimental results obtained with hypertonic C-Med are given for shorter repair intervals. For comparison calculated curves for a saturated and an unsaturated repair system as explained in Appendix I are shown also in Fig. 8a. As can be seen, the experimental data agree much better with a saturated system.

The survival at long repair times, S_∞, was determined to be $S_\infty = 0.30$ with an accuracy of about 5 to 10%. Therefore additional curves for N_B/N_{B0} assuming $S_\infty = 0.32$ and $S_\infty = 0.28$ were determined from the same experimental data. There is a small influence on the initial shape of the curves for N_B/N_{B0}, but the time constant for the exponential decrease is rather uncertain, in this case $t_{0.37} = 8 \pm 2$ hr.

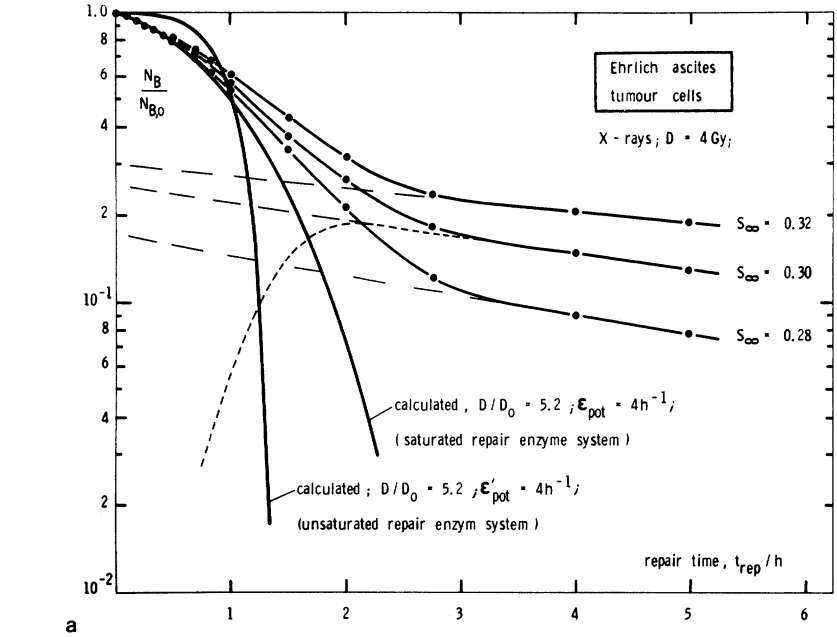
The difference between the curve for $S_\infty = 0.30$ and the calculated curve for a saturated repair system is given in Fig. 8a as a broken line. It indicates the production of slowly reparable lesions in the cells from those which are not repaired by the fast system.

The possibility that fast repair is exhausted after about 1 to 2 hr of operation was disproved by the following experiment: Cells were irradiated with $D = 4$ Gy and were allowed to repair for 1 hr. The repair of one-half the population was followed and further corresponded to the data shown in Figs. 8a and b. The other half of the population was irradiated once more with $D = 4$ Gy. The measured repair curve after the second irradiation was again biphasic with exactly the same repair frequency as that for the first irradiation. The time constants for slow repair were also identical, but the fraction of slowly repairing cells was increased by those left unrepaired from the first irradiation. Thus it seems certain that the shape of repair curves (Fig. 8) is not due to exhaustion of the repair system. The change from a quickly reparable lesion to a slowly reparable one might be due to thermal molecular movement, but this assumption requires experimental verification.

In Fig. 8b the initial part of the repair curve for cells in state B is drawn in a linear plot from results of an experiment with short repair intervals. As can be seen the experimental data can be represented within experimental uncertainties by a calculated curve (see Appendix I) with either $\epsilon_{pot} = 5\ h^{-1}$ corresponding to a repair time of $t = 12$ min for the elimination of one lesion or by $\epsilon_{pot} = 4\ h^{-1}$ corresponding to a repair time of 15 min. The repair time for the fast repair system therefore is uncertain within about 20%.

Experiments with the same cells (*27*) in which repair of PLD was inhibited by application of β-arabinofuranosyladenine (ara-A) have also produced an exponential dose–survival curve which had about the same slope as that measured here within the given experimental uncertainties. Also a fast and a slow repair component could be determined with about the same time constants. This indicates that the repair process analyzed here can be confirmed also with quite different experimental methods.

Repair of PLD in nutrient medium. Plateau-phase ascites cells were prepared and irradiated as described above. Instead of incubation in C-Med after irradiation, these cells were incubated in F-Med. At certain intervals after irradiation hypertonic treatment was performed as described and cell survival was measured by



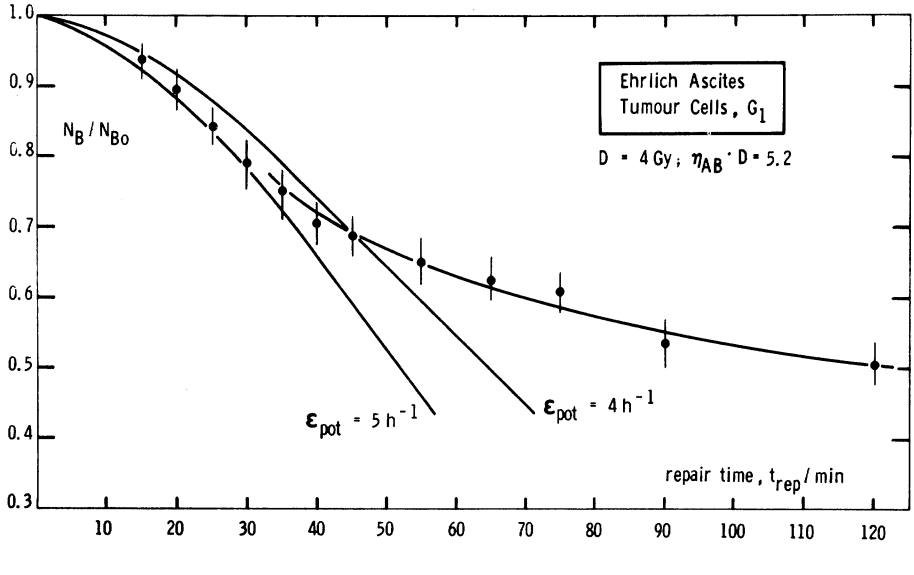


FIG. 8. Decrease of relative number of cells in state B with PLD as a function of repair time in conditioned medium at 37°C. (a) Experimental data for different values S_∞. Comparison with calculated curves for saturated and unsaturated repair systems. Broken line: experimental values for S_∞ = 0.30 minus calculated values for a saturated repair system. (b) Calculated curves for different ϵ_{pot} using Eq. (I.9). (Uncertainty of the experimental values: two standard deviations of colony counting.)

colony-forming ability on nutrient agar. The increase in cell survival due to repair in F-Med measured on the same day as repair in C-Med is shown in Fig. 9a. The curves differ in the final value at $t_{rep} = 20$ hr, indicating that there is less repair in F-Med. The quantitative analysis of the reduction of cells in state B shows that the repairable damage in F-Med is eliminated faster than that in C-Med (Fig. 9b). From the fact that the final repair in F-Med is less than that in C-Med it is obvious that some potentially lethal lesions are fixed during the repair time and in this way lead to a reduction of the relative number of cells in state B. The quotient of (N_B/N_{B0}) for C-Med and F-Med is drawn also in Fig. 9b; the resulting straight line in this semilogarithmic plot agrees with the assumption of a time constant ϵ_{BC} for fixation of PLD in F-Med which is independent of repair time. Therefore at least for the slow repair of PLD fixation of these lesions or transformation into an irreparable state C has to be considered and is included in the graph in Fig. 9a.

The question arises of whether fixation of PLD can be neglected for repair in C-Med or whether it can be made responsible for the difference between N_A/N_{A0} for $t_{rep} \rightarrow \infty$ and the production of irreparable damage as indicated by the broken line in Fig. 6 (see also Appendix II). Measurements of repair of PLD have therefore been done in C-Med with additions of F-Med. Even small fractions of F-Med decrease the final survival value, and a solution with 50% F-Med has the same effect as pure F-Med. The difference in final survival is equivalent to an addition of about 2–5% F-Med in the C-Med. Such a portion cannot be excluded if cells are grown into the plateau phase at $t = 72$ hr (see Fig. 1). It was found experimentally (*11*) that cells from the late plateau phase ($t = 120$ hr in Fig. 1) are able to repair to a higher survival level than cells from the early plateau phase ($t = 72$ hr). This agrees with the assumption that certain ingredients of the nutrient medium, possibly growth factors in the serum component, are used up by the cells in the plateau phase. Therefore for cells from the early plateau phase a certain fixation of PLD in C-Med should be expected, having a time constant of about $\epsilon_{BC} = 0.03$ h^{-1} to explain the difference between survival after long repair time and production of irreparable damage according to the broken line in Fig. 6.

The final survival after repair in F-Med is identical with the survival found for the usual immediate plating (IP) of irradiated cells on nutrient agar or in liquid nutrient medium (see Fig. 6). It must therefore be concluded that dose–survival curves for plateau cells obtained in the usual way are mainly a result of competition between repair of PLD and fixation of such lesions as irreparable damage. Both reactions take place on the nutrient agar after irradiation and depend on the physiological state of the cell, and their environment as can be seen from the data presented for F-Med and C-Med in Fig. 9a. Therefore a mathematical description of dose–survival curves for plateau-phase cells neglecting these biological reactions would be meaningless.

APPENDIX I

Repair of Poisson-Distributed Potentially Lethal Lesions

Lethality of cell damage measured by colony-forming ability can be the result of either a potentially lethal lesion (transition of a vital cell from state A to B) or

an irreparable lesion; the cell is then assumed to be in state C (*28*, *29*) [for a review of such models see (*30*)]. Simultaneous production of both types of lesions will be discussed in Appendix II. Here the production and repair of PLD in irradiated cells are discussed quantitatively for comparison with the respective experimental data given in this paper.

During production of PLD the number N_A of cells in state A (competent to grow to a macrocolony) is reduced due to irradiation with the absorbed dose fraction dD by

$$dN_A = -\eta_{AB} \cdot N_A \cdot dD, \tag{I.1}$$

where η_{AB} is the radiation reaction constant (unit: Gray $^{-1}$) for the transition of cells from state A to B.

The probability P_A of finding a cell in state A after irradiation with absorbed dose D can be obtained by integrating Eq. (I.1):

$$P_A = N_A/N_{A0} = e^{-\eta_{AB} \cdot D} = e^{-\bar{n}} = \mathcal{P}(\bar{n}, 0), \tag{I.2}$$

where N_{A0} is the cell number (or cell concentration) at $D = 0$, $\bar{n}$ is the mean number of potentially lethal lesions per cell and $\mathcal{P}(\bar{n}, 0)$ is the probability for a cell having no PLD ($n = 0$) from the Poisson distribution of this number n in individual cells $\mathcal{P}(\bar{n}, n)$, as will be discussed in detail.

If in a large population of cells a mean number of potentially lethal lesions $\bar{n}$ per cell is produced by irradiation, the actual number n in individual cells is distributed randomly and can be described by the Poisson distribution $\mathcal{P}(\bar{n}, n)$:

$$\mathcal{P}(\bar{n},n) = (e^{-\bar{n}} \cdot \bar{n}^n)/n! \,. \tag{I.3}$$

The probability P_A of having no PLD ($n = 0$) is then

$$\mathcal{P}(\bar{n}, 0) = e^{-\bar{n}} \tag{I.4}$$

(since $n^0 = 1$ and $0! = 1$).

The product $\eta_{AB} \cdot D$ therefore is equal to the mean number $\bar{n}$ of potentially lethal lesions produced per cell [see Eq. (I.2)]. Correspondingly the probability P_B of finding a cell with one or more potentially lethal lesions is

$$P_B = 1 - P_A = 1 - e^{-\eta_{AB} \cdot D} = 1 - \mathcal{P}(\bar{n}, 0). \tag{I.5}$$

Repair of PLD by a saturated repair system. If the number of radiation lesions produced in a living cell is much larger than the available repair enzyme molecules, the system is saturated and the repair velocity is limited by the constant (small) number of repair enzyme molecules. The same fraction dn of potentially lethal lesions is then repaired per interval dt and is equal to the repair frequency ϵ_{pot} for these lesions:

$$\frac{dn}{dt_{rep}} = -\epsilon_{pot}. \tag{I.6}$$

This mechanism of repair and the transition of cells from state B to A can be explained using a quantitative example shown in Fig. 10.

If each cell of the population has received a mean of $\bar{n} = 1$ lesion, then the

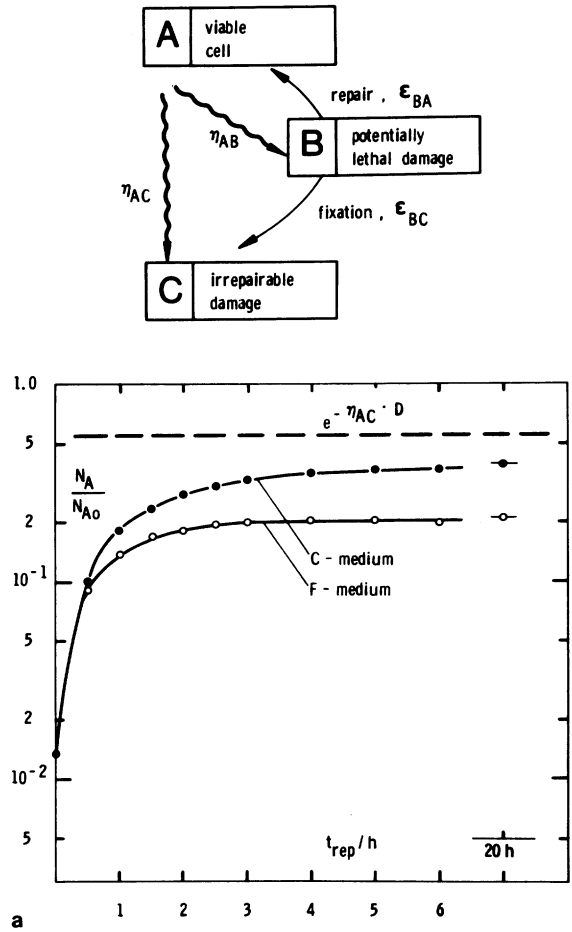


FIG. 9. (a) Repair of PLD in C-Med and F-Med at 37°C after irradiation with $D = 4$ Gy. Hypertonic treatment after t_{rep} with hypertonic C-Med. (b) Decrease of relative cell number in state B as a function of repair time in C-Med at 37°C (●) and in F-Med at 37°C (○). (■) is the quotient of the experimental values for C-Med and F-Med.

fraction of cells without damage is $\mathcal{P}(1, 0) = 0.37$. After a repair time t_{rep} such that $\epsilon_{pot} \cdot t_{rep} = 1$, just one potentially lethal lesion is repaired in each cell. The number of cells in state A is then increased by those cells having only one potentially lethal lesion $\mathcal{P}(1, 1) = 0.37$. After $\epsilon_{pot} \cdot t_{rep} = 2$ the number N_A is increased by $\mathcal{P}(1, 2)$ and so on. In this way in Fig. 10 the population of cells in state B is divided into fractions corresponding to these Poisson distributions; survival curves for N_A/N_{A0} are drawn for increasing values of $\epsilon_{pot} \cdot t_{rep}$ in Fig. 11. As can be seen an initial shoulder is reproduced very quickly with the final slope of the curve parallel to the exponential curve for $\epsilon_{pot} \cdot t_{rep} = 0$. For larger values of $\epsilon_{pot} \cdot t_{rep}$ this slope decreases and finally for $t_{rep} \rightarrow \infty$ the line $N_A/N_{A0} = 1$ is reached.

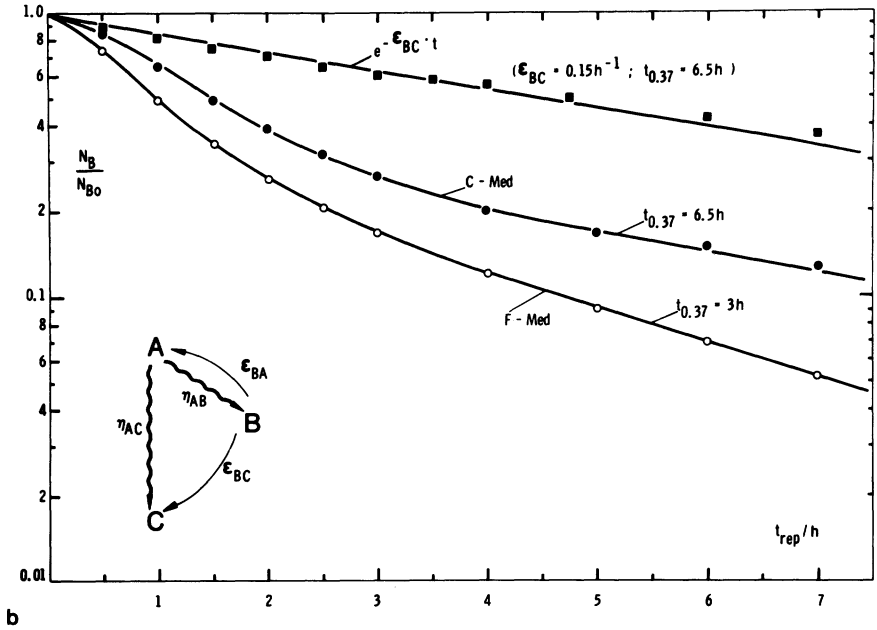


FIG. 9.—*Continued.*

Quantitatively the probability for a cell in state A after a repair time t_{rep} is given by

$$P_A(t_{rep}) = \sum_{n=0}^{\epsilon_{pot}\cdot t_{rep}} \mathcal{P}(\bar{n}, n) \tag{I.7}$$

and the probability for a cell remaining in state B is

$$P_B(t_{rep}) = 1 - \sum_{n=0}^{\epsilon_{pot}\cdot t_{rep}} \mathcal{P}(\bar{n}, n). \tag{I.8}$$

For the quantitative analysis of repair kinetics such as we have done it is useful to normalize the probability $P_B(t_{rep})$ of cells in state B after repair time t_{rep} to the probability P_B of these cells at $t_{rep} = 0$ [see Eq. (I.5)]:

$$\frac{P_B(t_{rep})}{P_B} = \frac{1 - \sum^{\epsilon_{pot}\cdot t_{rep}} \mathcal{P}(\bar{n}, n)}{1 - \mathcal{P}(\bar{n}, 0)} = \frac{N_B}{N_{B0}}. \tag{I.9}$$

Numerical values for $P_B(t_{rep})/P_B$ calculated using Eq. (I.9) are given in Fig. 12a as a function of the dimensionless parameter $\epsilon_{pot}\cdot t_{rep}$. Such curves can be used for comparison with experimental results (see, for example, Figs. 7 and 8) independent of the presence of irreparable lesions as will be shown later [see Eq. (II.4)].

Repair of PLD by an unsaturated repair system. If the number of repair enzyme molecules in a cell is much larger than the number of lesions produced, the repair

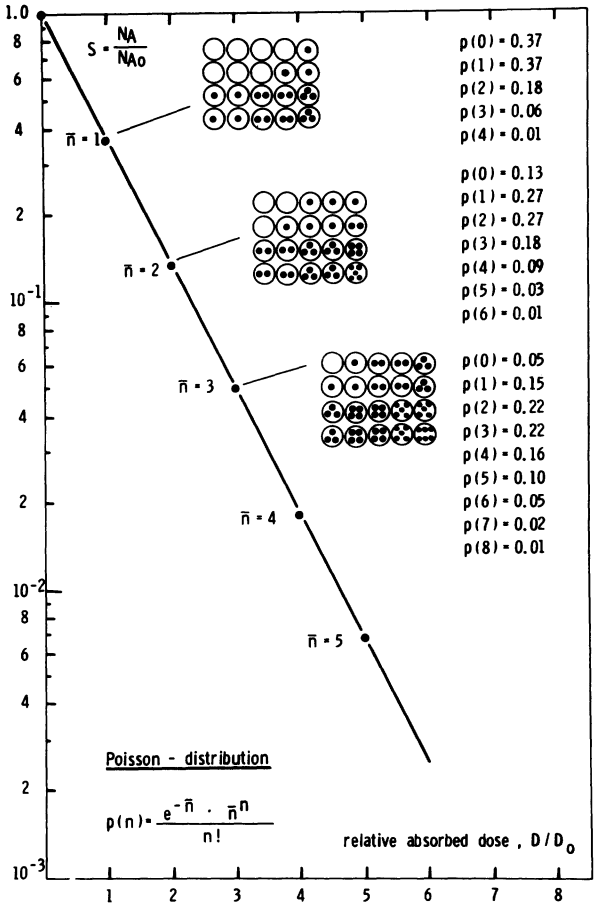


FIG. 10. Poisson distributions $\mathcal{P}(n)$ of the number of potentially lethal lesions per cell for different relative absorbed doses D/D_0. D_0 is the absorbed dose where the mean number of potentially lethal lesions per cell is $\bar{n} = 1$; $\mathcal{P}(0)$ is identical with the survival $S = N_A/N_{A0}$.

velocity (or repair frequency) depends on the number of unrepaired lesions.

$$\frac{dn}{dt_{rep}} = -\epsilon'_{pot} \cdot n. \tag{I.10}$$

Assuming that a cell belongs to state A if n is larger than $n = 0.37$, the curves in Fig. 12b have been calculated corresponding to Fig. 12a and can be used for comparison with experimental results (see Fig. 8a). The above-mentioned assumption of $n = 0.37$ omits uncertainties for cells which have received only one potentially lethal lesion by irradiation and the curves for larger absorbed doses are not influenced significantly by this assumption.

Repair of PLD limited by stochastic reactions. The repair of PLD in cells can be limited by stochastic reactions such as the Brownian movement of molecules. In this case each cell in state B stays in that state for a certain time, or in other

words, a certain repair time constant ϵ_{BA} exists for retransition into state A. Change of cell number in state B can then be expressed by

$$dN_B = -\epsilon_{BA} \cdot N_B \cdot dt_{rep} \tag{I.11}$$

or

$$N_B = N_{B0} \cdot e^{-\epsilon_{BA} \cdot t_{rep}} \tag{I.12}$$

which results in a straight line if N_B/N_{B0} is drawn in a semilogarithmic plot as a function of t_{rep}.

APPENDIX II

Dose–Survival Curves due to the Production of Different Types of Lesions

In general, energy deposition by ionizing radiation produces repairable (potentially lethal) and irreparable damage in living cells, most probably due to molecular

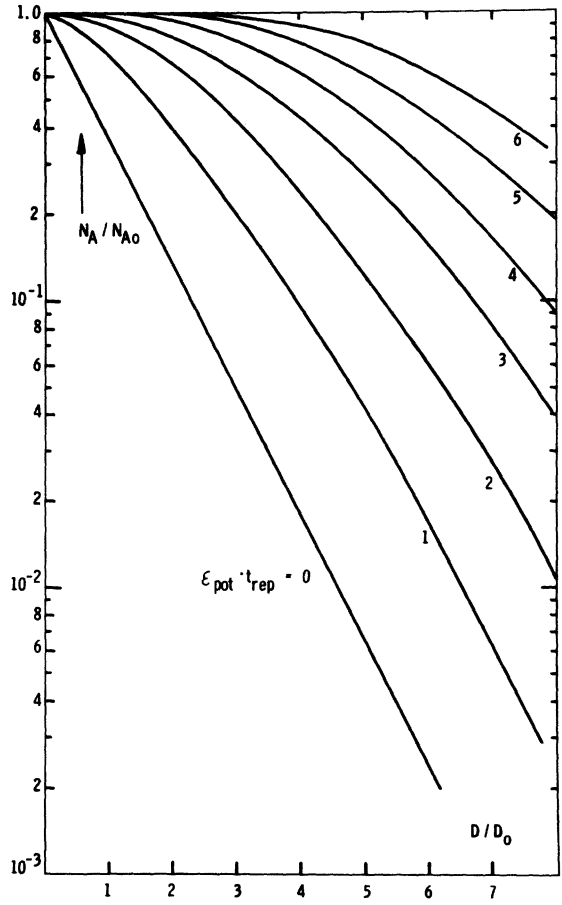


FIG. 11. Calculated dose–survival curves for different repair intervals $\epsilon_{pot} \cdot t_{rep}$, where ϵ_{pot} is the repair frequency of the saturated repair system.

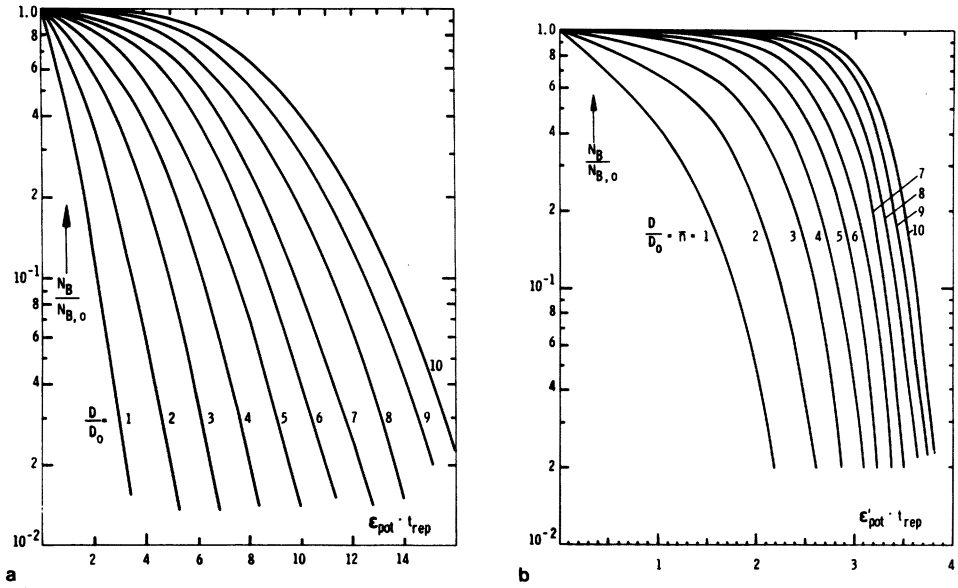


FIG. 12. (a) Calculated decrease of relative cell number in state B with PLD using Eq. (I.9) for a saturated repair system. (b) Calculated decrease of cells in state B with PLD for an unsaturated repair system.

reactions in the DNA. Therefore three different states of a living cell have to be assumed:

state A: cell capable of producing a macrocolony;

state B: cell with reparable damage;

state C: cell with irreparable damage.

The transition from state A to B has been discussed in Appendix I; corresponding to Eq. (I.1) the transition into state C can be described by

$$dN_A = -\eta_{AC} \cdot N_A \cdot dD. \tag{II.1}$$

The probability for a cell in state C is [see Eq. (I.5)]:

$$P_C = 1 - P_A = 1 - e^{-\eta_{AC} \cdot D}, \tag{II.2}$$

where η_{AC} is the radiation reaction constant for the transition from A to C.

Both lesions, the reparable and irreparable, may occur independently of each other. Therefore the probability for a cell remaining in state A is

$$P_A = \frac{N_A}{N_{A0}} = (1 - P_C) \cdot (1 - P_B). \tag{II.3}$$

Using Eq. (II.2) for P_C and Eq. (I.8) for P_B this results in

$$\frac{N_A}{N_{A0}} = e^{-\eta_{AC} \cdot D} \cdot \sum_{n=0}^{\epsilon_{pot} \cdot f_{rep}} \mathcal{P}(\bar{n}, n), \tag{II.4}$$

where $\bar{n}$ is the mean number of potentially lethal lesions: $\bar{n} = \eta_{AB} D$. Equation (II.4) describes dose–survival curves as in Fig. 11 but with an initial slope determined by η_{AC} (see Fig. 6).

Two extreme conditions can be approached experimentally

$$t_{rep} = 0: \quad \frac{N_A}{N_{A0}} = e^{-\eta_{AC} \cdot D} \cdot \mathcal{P}(\bar{n}, 0) = e^{-(\eta_{AC} + \eta_{AB}) \cdot D}, \tag{II.5}$$

$$t_{rep} \to \infty: \ (D \to 0): \quad \frac{N_A}{N_{A0}} = e^{-\eta_{AC} \cdot D}. \tag{II.6}$$

Experiments performed under these conditions can be used to determine η_{AC} and η_{AB}. Analysis of repair kinetics as explained in Appendix I can be made independently of the presence of irreparable damage.

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