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University of California

Ernest O. Lawrence Radiation Laboratory

SUPPLEMENT TO BIOLOGY AND MEDICINE
SEMIANNUAL REPORT
Spring 1964

Berkeley, California

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UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
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Contents

ON THE SPATIAL DISTRIBUTION OF RADICALS PRODUCED BY IRRADIATION[*]

S. J. Wyard[†]

Introduction

The possibility of using electron spin resonance techniques to measure the spatial distribution of radicals appears to have been first suggested by Livingston,[1] in a paper given at the First International Congress of Radiation Research in 1958. However, the method has not found much application because, although radicals can be trapped in many materials in the solid state, they cannot be trapped sufficiently closely together for the ESR method to be used. The reasons for this are not known.

While working with frozen solutions of hydrogen peroxide, Dr. Smith and I discovered that this material is an exception to the rule just mentioned, and that radicals can be trapped quite close together in it - the closest distance observed being about 4 Å.[2] So frozen solutions of hydrogen peroxide provide a suitable working substance for measuring spatial distributions of radicals; and we published a preliminary report on this in 1961.[3] I am at present continuing these measurements, using the facilities provided by Professor Tobias at the Donner Laboratory; and although this work is not yet complete, I thought it might be of sufficient interest to give an interim report at this meeting.

Method

There is not time to go into the details of the method, but I should mention that there are in fact two methods. The first depends on the linewidth of the normal ESR spectrum at $g = 2$, while the second depends on the intensity of a so-called forbidden transition which occurs at $g = 4$, i.e., at half the magnetic field. Both methods give essentially the same information, but it is useful to have both as a check on each other. If the radicals are distributed throughout the sample in pairs, clusters, or columns, then the method gives the average spacing between radicals within individual pairs, clusters, or columns. The spacing can be obtained from the measurements via theoretical calculations; alternatively the methods can be calibrated by producing known randomly spaced concentrations of radicals.

Figure 1 shows the buildup of radical concentration with radiation dose. At 77°K the radicals are stable for months. The maximum concentration of radicals shown in Fig.1 is about 2% of the number of molecules, and the average spacing at this concentration is about 12 Å.
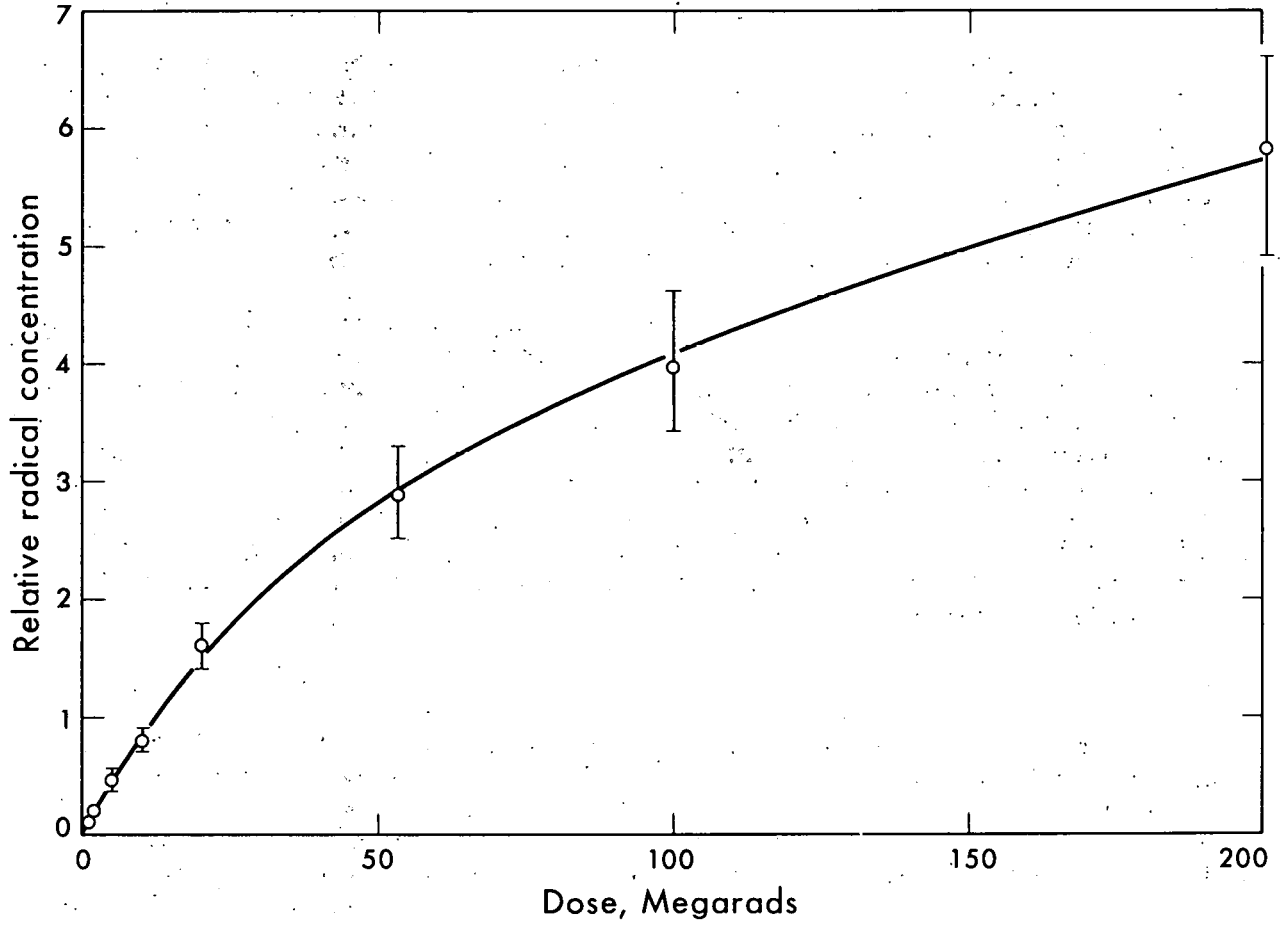
Fig. 1. Dose-effect curve; 50% w/w H_2O_2—H_2O; 77°K; 6.5-MeV electrons.

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Figure 2 shows the increase in line width with radiation dose for the same conditions as Fig. 1. By comparing Figs. 1 and 2 the variation of line width with radical concentration is obtained. The separation between salient points on the spectrum can be measured with sufficient accuracy that a difference in radical concentration of 3×10^{-3} M should be detectable. However, the line shape is complex, which makes it more difficult to extract the line width, and at present the limit of resolution is about 10^{-2} M.

Figure 3 shows the ratio of intensities of the g = 4 and g = 2 spectra plotted against radiation dose, for the same conditions as Figs. 1 and 2. The intercept at zero dose, which is obtained by extrapolation, gives the initial concentration of radicals in clusters. With this method the limit of resolution is also about 10^{-2} M, a figure which can probably be improved on.

The initial local concentrations may be removed by annealing the samples. The radicals diffuse rapidly at about 140°K; annealing for a few minutes at this temperature produces a sample in which the radicals are essentially separate. Such a sample provides a base line for the measurement of line widths.

Results

These methods have been used to study the spatial distribution of radicals when they are produced by either uv or ionizing radiation.

With uv radiation the radicals are produced in pairs. The average separation varies according to the conditions of radiation from about 5 to 10 Å. The average separation is greater with shorter-wavelength radiation (wavelengths between 3500 and 2.537 Å were used), is greater when irradiations are carried out at 77°K than at 4.2°K, and also increases with radiation dose.

Turning to ionizing radiation, the local concentrations are the same for all the low-LET radiations used, from 6.5-MeV electrons to 16-MeV deuterons (average LET from 0.6 to 14 keV/μ). The local concentration is close to the limit of what can be measured, and is estimated at 0.05 M. The corresponding average separation is 32 Å. In an early experiment a higher local concentration was obtained by using α particles.[3] This result might be criticized, because the irradiation was carried out with thorium X, and this introduced thorium X, chloride ions, and an acidic solution into the sample; these might have affected the measurements. An experiment is now in progress using heavy ions, and the results appear to confirm the α-particle experiment. Preliminary values for local concentrations using heavy ions are 0.08 M with helium (average LET 35 keV/μ) and 0.1 M with carbon (average LET 310 keV/μ).
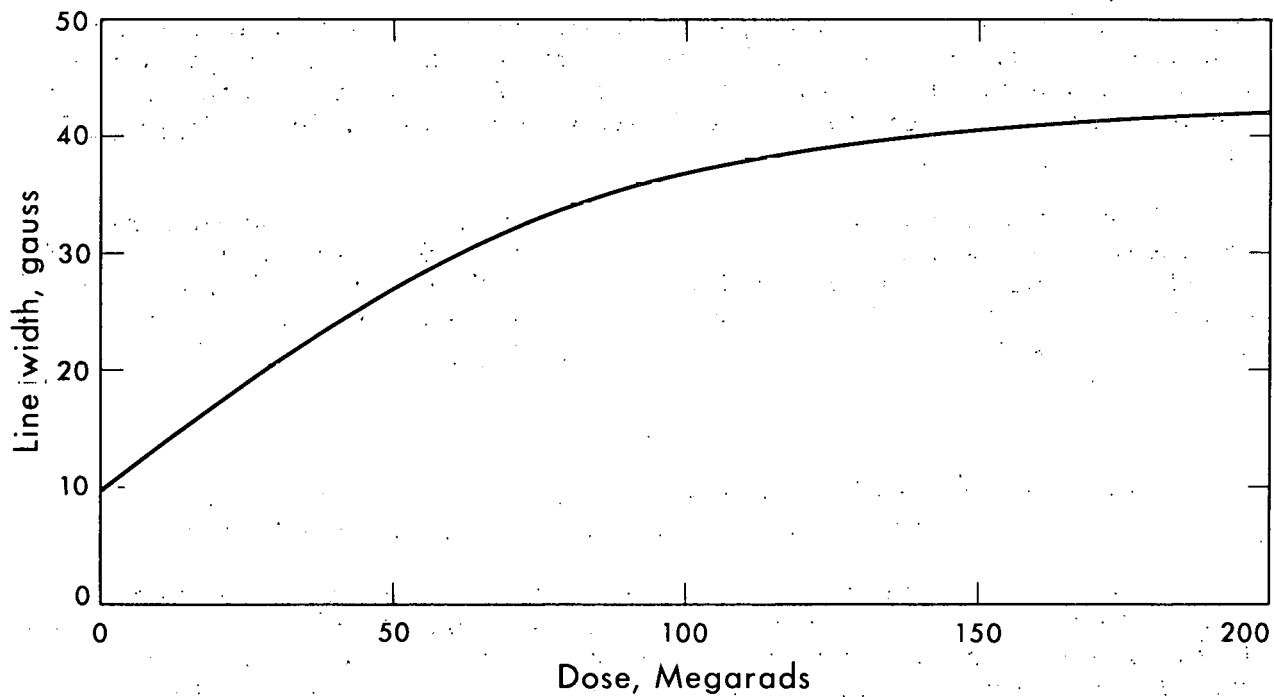
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Fig. 2. Line width variation; 50% w/w $H_2O_2-H_2O$; 77°K;
6.5-MeV electrons.
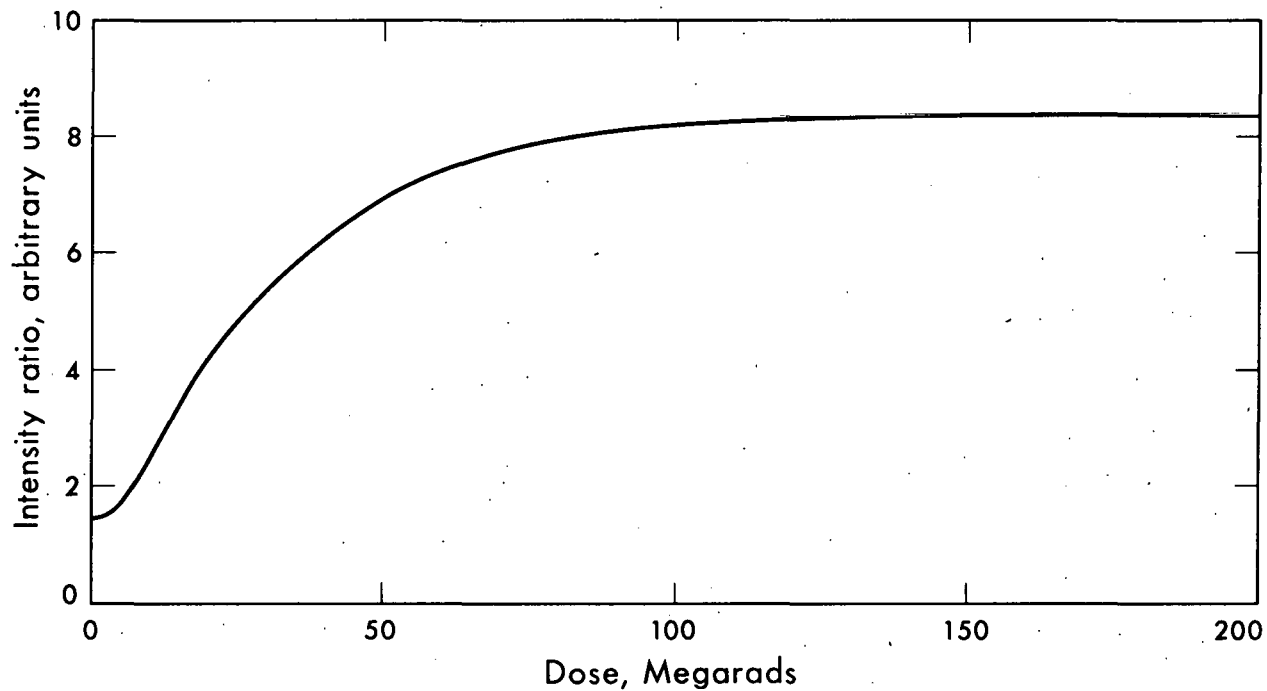
Fig. 3. Intensity ratio (g = 4 to g = 2); 50% w/w H_2O_2-H_2O; 77°K; 6.5-MeV electrons.

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Discussion

Taking the results for uv radiation first, the H_2O_2 molecule is split into two fragments which can possess kinetic energy equal to the difference between the quantum energy of the uv photon and the energy of the bond which has been broken. With a shorter wavelength there will be more kinetic energy, leading to a greater diffusion apart. This accounts for the observed variation in separation with wavelength. The variation in separation with temperature of the sample is also reasonable, since at 4.2 °K the glass would be more rigid than at 77 °K, so that a given excess energy would result in less diffusion. The increase in separation with increasing dose is more surprising; in fact, one would expect that as the concentration of radicals built up the pairs would begin to overlap, resulting in a smaller average separation. The fact that the opposite occurs might be explained by a local heating effect. The yield is 1 radical for every 15 photons absorbed, so that in the majority of cases the two fragments of the photolyzed molecule recombine, and all the energy is transferred to the surrounding material in the form of heat. For glassy samples this causes a temporary softening (at 140 °K the glass is sufficiently soft that radicals diffuse rapidly), and would allow an existing pair of radicals in the neighborhood to diffuse a little farther apart. Another possibility is that if a pair of radicals is formed close to an already existing pair, the two closest radicals may combine, leaving the other two farther apart.

Turning to the results for ionizing radiation, the variations of local concentrations are what one would expect, since the concentrations are low for low-LET radiations where the clusters are separated, and increase with LET above the value of LET for which the clusters begin to overlap. However, the absolute values of concentration are quite low. The yield is of the order of 10 radicals per 100 eV, from which it may be calculated that the radicals formed by the deposition of 100 eV occupy a volume equal to a sphere 90 Å in diameter. In the radiation chemistry of water it is often assumed that a cluster or spur of radicals formed by 100 eV occupies a sphere of about 25 Å diameter.[4, 5] The first possibility to consider, to account for this difference, is that the radicals are initially formed in clusters of 25 Å diameter in the hydrogen peroxide glasses, but diffuse to a volume 90 Å in diameter before being stabilized. The results for uv radiation, discussed above, suggest that for ionizing radiation also, the radicals are trapped a little distance from the site of the ionized molecule, but not far enough to account for the difference between 25 and 90 Å. Moreover, if there is appreciable diffusion of radicals, either because they possess kinetic energy or because of local heating, then irradiation at a lower temperature should produce clusters occupying a smaller volume with correspondingly higher local concentrations. The few measurements I have made so far at 4.2 °K are inconclusive, but it does appear that irradiation by electrons at 4.2 °K followed by measurement at 77 °K produces exactly the same spectrum as irradiation and measurement both at 77 °K. Hence the observed local concentrations may be very close to the initial ones at the moment of formation. Whether or not a 100-eV cluster should occupy the same volume in frozen solutions of hydrogen peroxide as in liquid water, I don't know.

It is also possible to obtain some information about the volume oc-
cupied by a cluster from the buildup of radical concentration with dose. If
it is assumed that a given amount of absorbed energy affects a certain
restricted volume, then the total volume affected increases with dose,
following an exponential curve. The observed buildup of radicals (Fig. 1)
follows a curve which is approximately exponential; from this one can calcu-
late a minimum value for the volume affected by the energy packet. For
frozen solutions of hydrogen peroxide this calculation gives a minimum
value equal to a sphere of 35 Å diameter for a 100-eV cluster. The previous
discussion suggests that the volume is considerably larger than this; how-
ever, even this minimum value is larger than the value often assumed in the
radiation chemistry of water.

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     diameter of a typical spur is less than 40 Å.

EPR STUDIES ON OH RADICAL IDENTIFICATION IN IRRADIATED H₂O

T. E. Gunter* and C. D. Jeffries†

After the identification of atomic hydrogen in irradiated acids at 77°K by Livingston et al.[1] and in neutral ice at 4.2°K by Piette et al.[2] it became popular to attribute the rather poorly resolved EPR spectrum in irradiated polycrystalline ice at 77°K, and sometimes that of ice irradiated at 4.2°K, to the OH radical. This was a good guess as to the identity of the radical species in the cases mentioned above, but real proof that the OH radical is formed when H₂O is irradiated was not provided by the experiments with polycrystalline ice.

The first contribution of the present study to this field came when single crystals of ice irradiated at 77°K were studied in detail by EPR at both 77°K and 4.2°K. In these studies an effective spin Hamiltonian was sought which took account of Zeeman and hyperfine interactions. Figure 1 shows, as an example of these spectra, the spectra measured at both 77°K and at 4.2°K of a single crystal of ice irradiated at 77°K, both spectra taken at the same orientation in the plane of hexagonal symmetry.

Whereas in past work quite different spectra were found for polycrystalline ice irradiated and studied at 77°K and ice irradiated and studied at 4.2°K (each spectrum similar to its single-crystal counterpart shown in Fig. 1), it was usually assumed that only one of them could be the OH radical. The study presented here showed that the form of the spectrum of irradiated ice goes reversibly (without change) between the previously published 4.2°K and 77°K spectral forms. Hence it is likely that the spectra are caused by the same type of radicals. The reason for this spectral change does not appear to be a simple phase change, for both spectra showed hexagonal symmetry, as expected from tetrahedrally bonded ice. Also the spectra of crystals which had been irradiated at 77°K, cooled to 4.2°K, and later heated again to 77°K were of the single-crystal type, rather than the polycrystalline type. As well as could be determined, these spectra were identical to those of the same crystal taken after irradiation at 77°K but prior to cooling to 4.2°K.

The reason why the irradiated ice spectrum changes with temperature is still being studied; it is believed to be related to the splitting and populations of levels affected by crystal-field (hydrogen bond) interaction. Because there are many inequivalent sites in the ice lattice occupied by the radicals (seven, presumably, for OH), and because of the large number of protons surrounding each site and producing line broadening, it was found impossible to completely resolve the spectrum for the radicals formed in irradiated single-crystal ice.
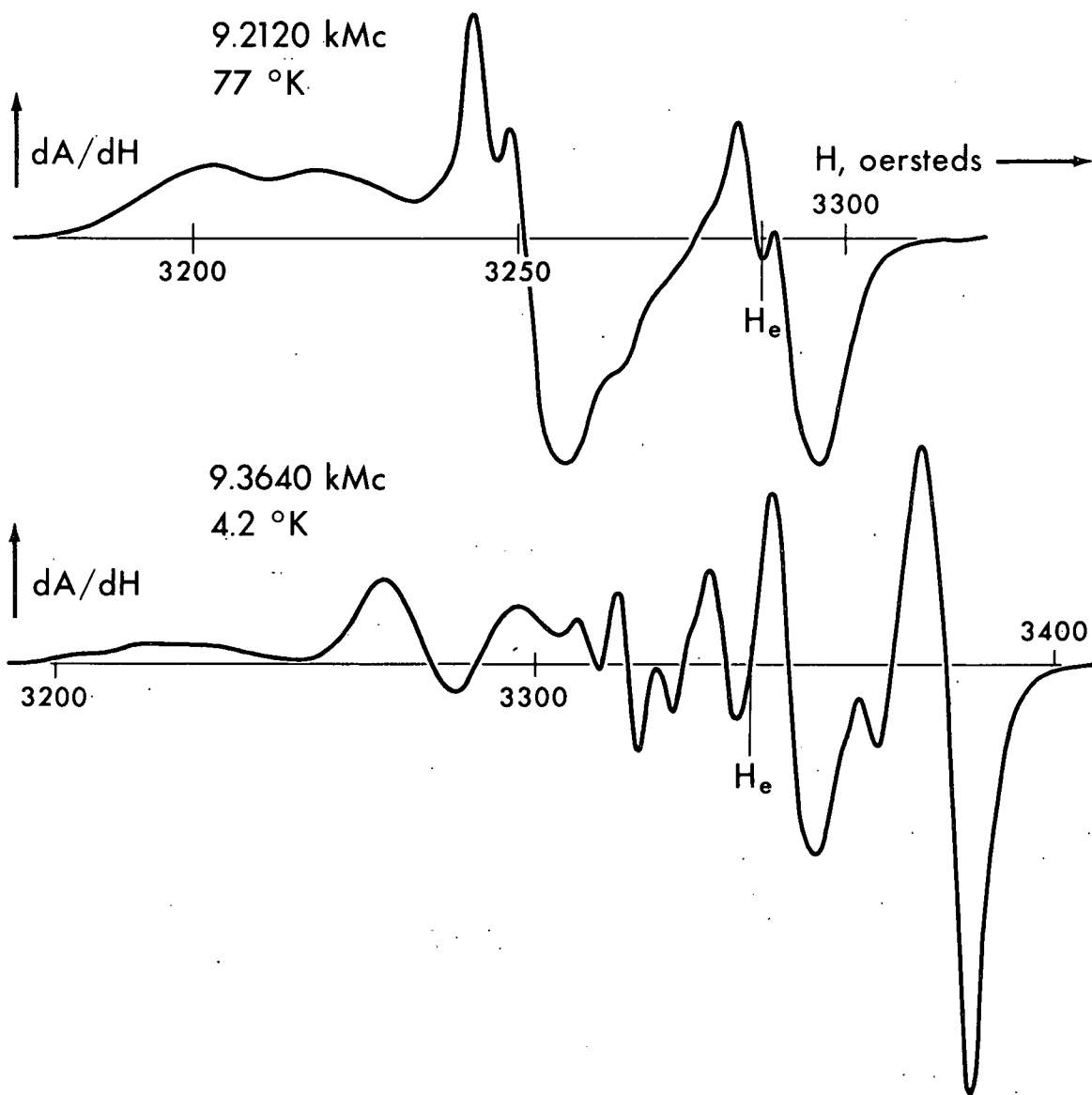
Fig. 1. Spectra of single-crystal ice irradiated by
electrons at 77°K and measured at 77°K and at
4.2°K.

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Irradiated H_2O was next studied in the form of water of crystallization in $CaSO_4 \cdot 2H_2O$. In this crystal the water occupies only two inequivalent sites, and the proton density is much less than in ice itself. The spectra of irradiated $CaSO_4 \cdot 2H_2O$ observed at 77°K show an anisotropic hyperfine doublet with two inequivalent sites and a weak isotropic line near the free-electron g factor. This is similar in appearance (though not in scale) to the spectra observed by Wigen and Cowen,[3] who used somewhat different irradiation procedures. We seek to identify the hyperfine doublet with the OH radical, and to observe that the isotropic spectrum is similar to that of the trapped electron seen by Henriksen.[4]

An example of the observed spectra of irradiated $CaSO_4 \cdot 2H_2O$ is found in Fig. 2. This spectrum was made with the dc magnet field almost perpendicular to the symmetry axis of the radical. In this orientation the two inequivalent sites give the same spectrum.

The observations for irradiated $CaSO_4 \cdot 2H_2O$ were fitted to a spin Hamiltonian of the form

$$\mathcal{H} = \beta \vec{S} \cdot \begin{pmatrix} g_\perp & 0 & 0 \\ 0 & g_\perp & 0 \\ 0 & 0 & g_{||} \end{pmatrix} \cdot \vec{H} + \vec{S} \cdot \begin{pmatrix} B & 0 & 0 \\ 0 & B & 0 \\ 0 & 0 & A \end{pmatrix} \vec{I} \quad ,$$

where β is the Bohr magnetron, H the magnetic field intensity, $\vec{S}$ the electron spin, and $\vec{I}$ the nuclear spin (S = 1/2, I = 1/2). Values for $g_\perp$, $g_{||}$, A, and B are given, along with a comparison of the data to the predicted line positions of the chosen spin Hamiltonian, on Fig. 3.

The effective spin Hamiltonian found for the anisotropic lines given by irradiated $CaSO_4 \cdot 2H_2O$ represents a paramagnetic species with nuclear spin 1/2, effective electron spin 1/2 (as would be expected from a quenched OH radical spectrum), and axial symmetry. OH is the only candidate radical species which meets all the requirements, for O_2H would not be expected to show axial symmetry for Zeeman and hyperfine interactions. The lack of axial symmetry in O_2H could be somehow masked by trapping interactions, however, so one cannot completely exclude it when identifying the source of the hyperfine doublet.

We qualitatively explain the relative g anisotropy and quenching in the above-mentioned samples as follows: For OH radical one would expect orders of interaction somewhat like those of the iron group of paramagnetic elements. That is, strong molecular or atomic forces "lock" the molecule or atom in place and lead to nonconservation of angular momentum. That means that L is not a good quantum number and the L part of the spin Hamiltonian is quenched. In ice the "locking" mechanism is a strong hydrogen bond and the quenching is essentially complete ($\Delta g/g$ cannot be greater than 0.5%). In $CaSO_4 \cdot 2H_2O$, on the other hand, the "locking" mechanism is crystalline fields and is relatively weaker. The quenching is consequently not complete (here $\Delta g/g = 5.1\%$).
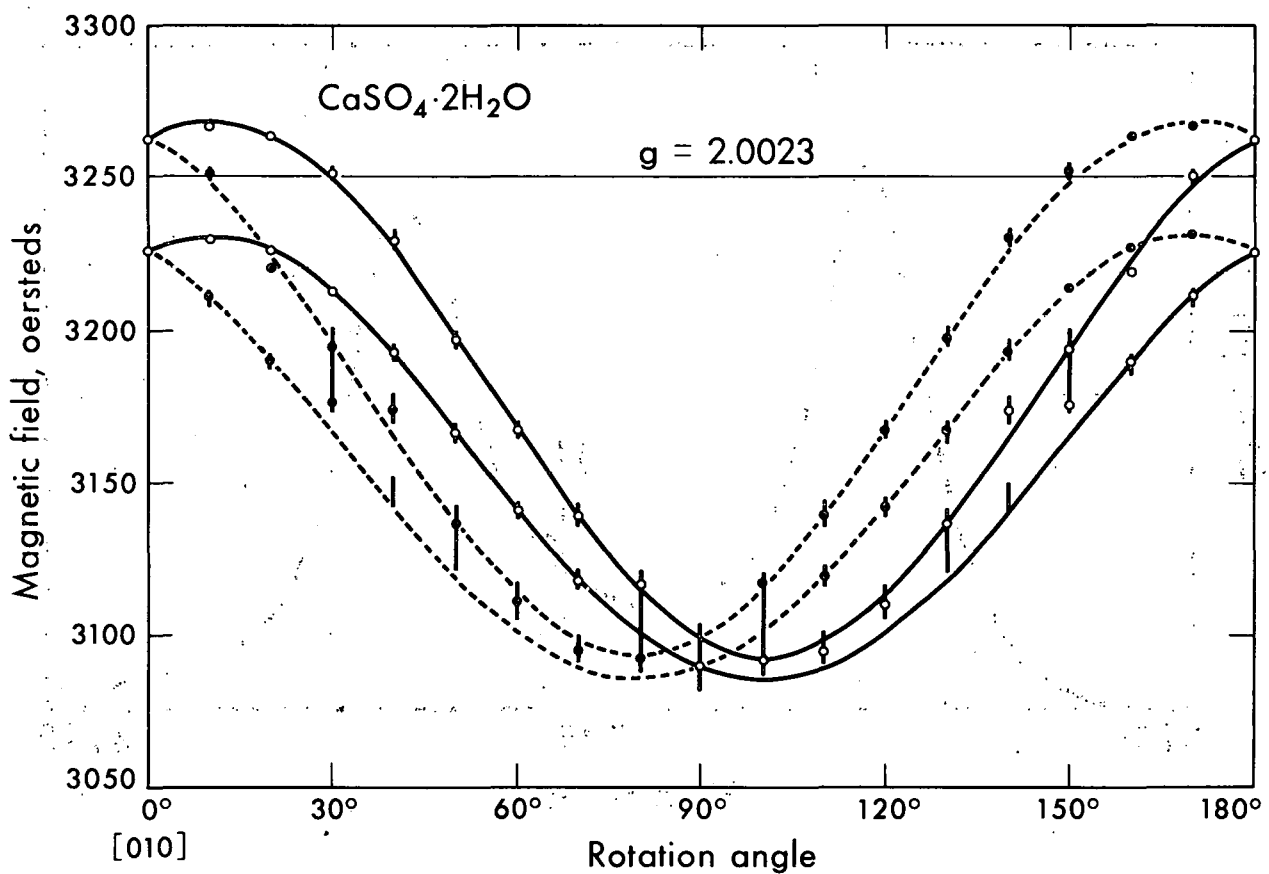
It may be possible after more study to explain the spectra of irradiated single-crystal ice by setting up a spin Hamiltonian with a hyperfine interaction similar to that found in irradiated $CaSO_4 \cdot 2H_2O$ with a Zeeman interaction giving less g anisotropy.
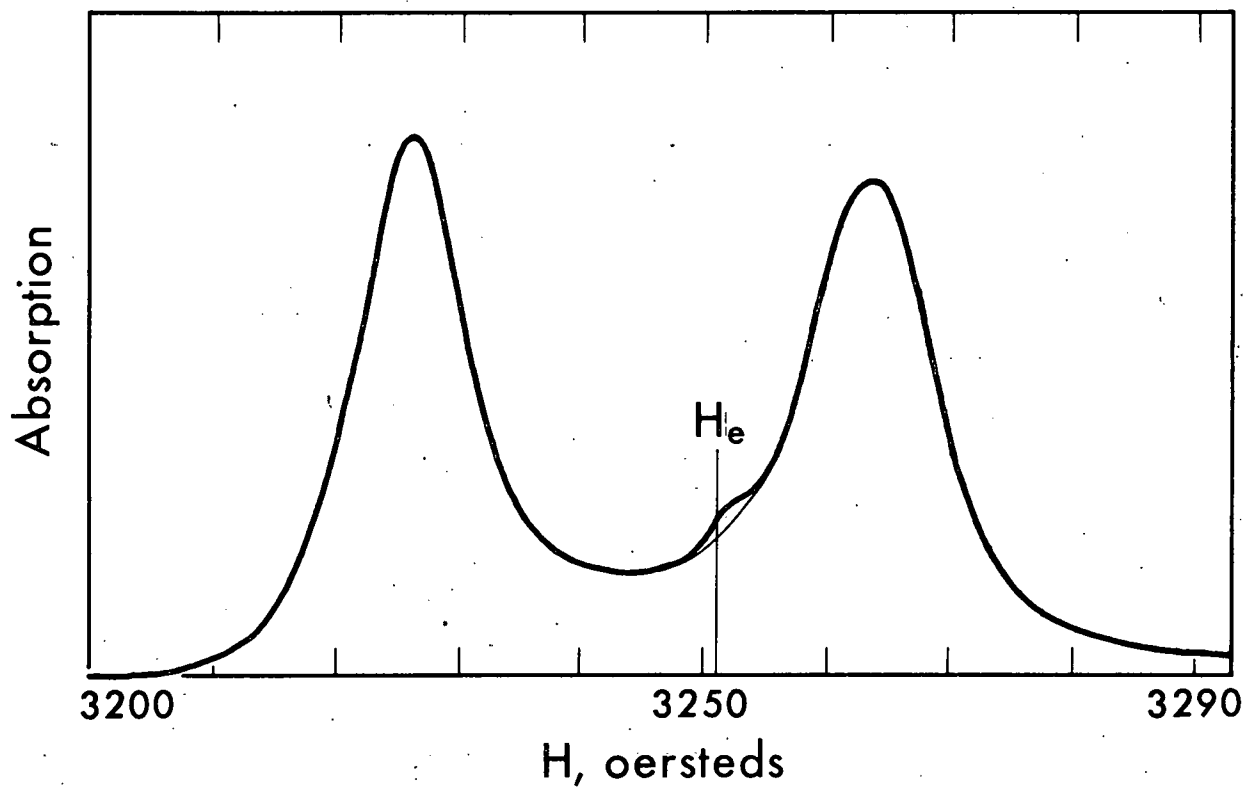
Fig. 2. Spectrum of $CaSO_4 \cdot 2 H_2O$ irradiated by electrons at $77°K$. $H \parallel [0\bar{1}0]$; 9.1110 kMc.

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Fig. 3. Spin Hamiltonian for irradiated $CaSO_4 \cdot 2 H_2O$.
$g_{||} = 2.1108$, $g_\perp = 2.0028$; A = 33.3 Oe, B = 37.9 Oe.

Presently work either in progress or planned includes:

(a) attempting to see atomic hydrogen spectra from the H atom in $CaSO_4 \cdot 2H_2O$ samples irradiated and observed at 4.2°K,

(b) studying the spectra of other simple hydrated crystals after irradiation, in which either Ca^{++} or SO_4^{--} or both are replaced by other ions.

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ELECTRON MICROSCOPY OF SINGLE-STRANDED DNA: CIRCULARITY OF φX-174 DNA

David Freifelder,[*] Albrecht K. Kleinschmidt,[†] and Robert L. Sinsheimer[‡]

The DNA of coliphage φX-174 is single-stranded[1] and apparently also circular.[2] This latter point, which is based upon the resistance of the DNA to digestion by exonucleases, is confirmed in this paper by electron microscopy.

Direct observation of DNA molecules by electron microscopy is possible if the DNA is adsorbed onto protein monolayers.[3] However, this has been complicated by entangling of a variable fraction of the molecules, although it is usually possible to find a sufficient number of extended, untangled filaments that a length distribution can be obtained.

When φX-174 DNA was examined, exceedingly severe tangling obviated any possible conclusion concerning length or configuration, although the success with the double-stranded replicating form[4] -- i.e., the clear demonstration of ring molecules[5,6] --strengthened the original surmise.

We have assumed that intramolecular hydrogen bonding is principally responsible for this problem and have therefore modified the preparation procedure to avoid it. This modification consists simply of denaturating the DNA by one of the methods of Freifelder and Davison[7,8] in order to break all hydrogen bonds, and subsequently handling the DNA in the presence of concentrations of formaldehyde which prevent hydrogen bond reformation.

DNA of φX-174 was prepared according to the method of Sinsheimer[1] and adjusted to a concentration of 70 μg/ml in 0.1 M NaCl–0.05 M Tris, pH 7.7, to serve as a stock. The solution for spreading was made by mixing the following components in sequence for the indicated standing times: 0.04 ml DNA + 0.04 ml 1 M NaOH, 20 sec; 0.4 ml 37% HCHO (adjusted to pH 11 with 1 N NaOH), 30 sec; 0.1 ml 1 M KH_2PO_4; 0.6 ml 0.01 M PO_4– 0.005 M versene, pH 7.8; 0.4 ml 4 M NaCl. One-tenth ml of the denatured DNA solution was diluted to 0.5 μg/ml by addition of 1 M NH_4 acetate containing 0.5% HCHO (neutralized and boiled for 10 minutes). Finally, 0.1 ml of 0.01% cytochrome C in 1 M NH_4Ac was added. This solution was spread 4 min later by the standard method,[3] except that the spreading was done on 0.5% HCHO (pretreated as above) instead of water. Lower concentrations of HCHO were not satisfactory. The visco-elastic properties and the spreading speed of the film on this solvent are comparable to those obtained with spreading on water. The film was transferred to carbonized support films on Pt grids by touching the surface, and the adhering droplet was removed by touching an ethanol surface.[5] After drying with filter paper, the preparation was rotary-shadowed with uranium at an angle of 6 to 10 degrees. Undenatured φX-174 DNA was also examined by the standard technique (without alkali and HCHO).

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Figure 1 shows representative single-stranded rings of ϕX-174 DNA. In a typical field about half of the DNA is easily seen as circular molecules; filaments account for fewer than 2%. The remainder of the DNA consists of molecules overlapping, aggregated, or tangled, which can be substantially reduced by spreading the DNA at lower concentrations. When mixed with other double-stranded material, single strands appear thinner, are more difficult to see because of lower contrast with the background and, in general, show sharp kinks, an observation consistent with the expected greater flexibility of the single strands. Undenatured preparations (untreated with alkali and HCHO) show badly tangled masses, yet without any visible free ends.

The lengths of most of the rings were measured by tracing a projected enlarged image on paper and laying 1/32-in. polyethylene tubing along the path. A histogram of the measured lengths of 186 rings is shown in Fig. 2. The mean contour length is 1.77 ± 0.13 μ (7.3%), and is greater than that found for the replicating form (1.70 μ ± 7%).[5] The greater mean length probably reflects the greater flexibility and lesser degree of coiling of single-stranded DNA, although some effect of HCHO is clearly possible. On the basis of a molecular weight of 1.7×10^6 [1] the mass per unit length of molecules prepared in this way is 0.95×10^6 daltons/micron, approximately half that found for double-stranded DNA.

The small number of filaments is an indication of the safety of the alkali treatment for denaturating DNA,[7] and furthermore reflects the stability of the linker causing ring closure. We assume that this is probably a covalent bond.[9] This is consistent with the resistance of infectious ϕX-174 DNA to alkali.[9]
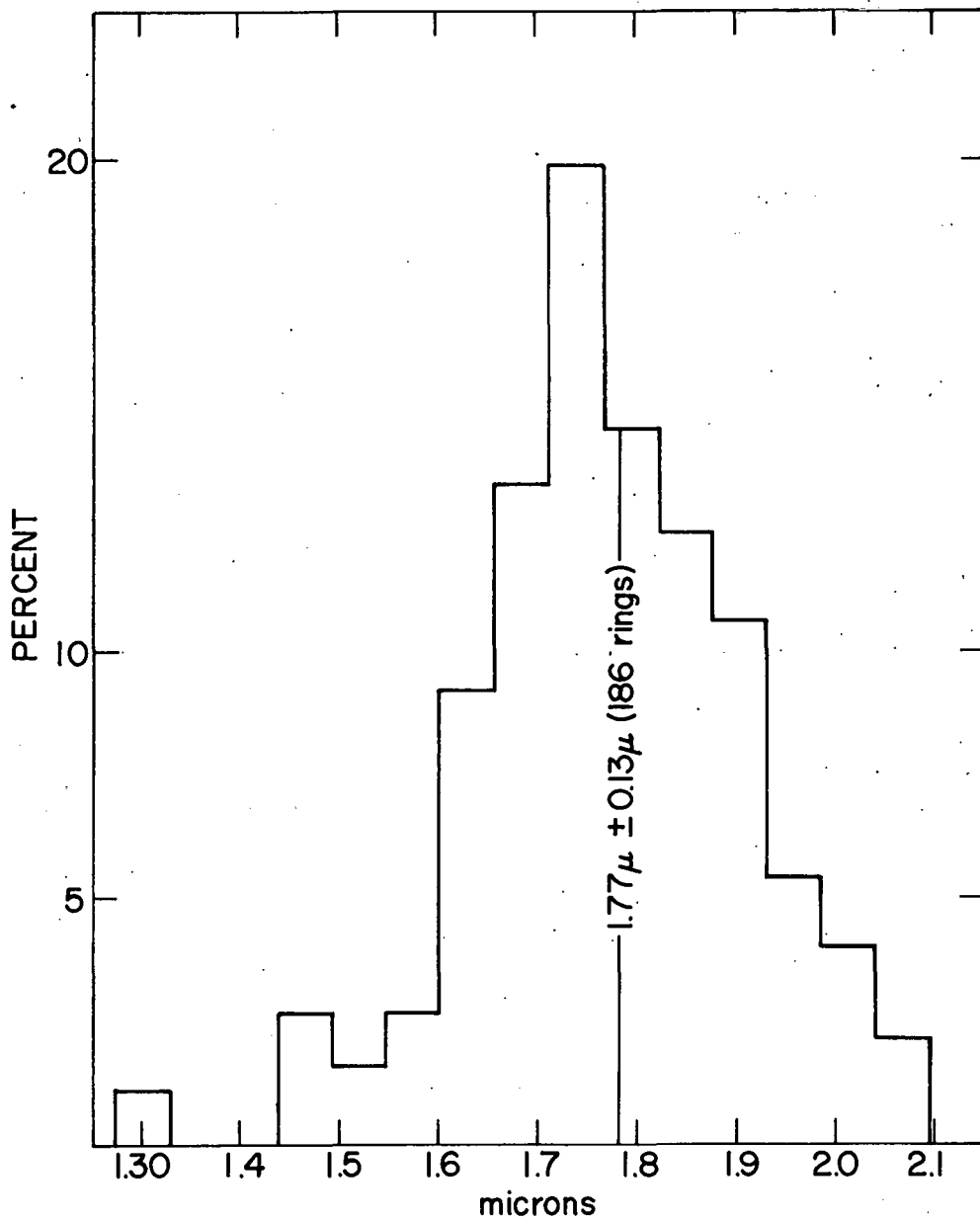
Further work is in progress using denatured single-stranded bacteriophage DNA derived from phages possessing double-stranded DNA.[10, 11]
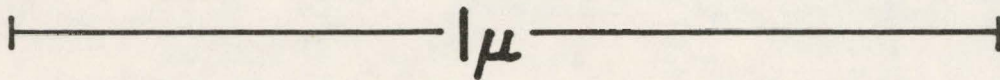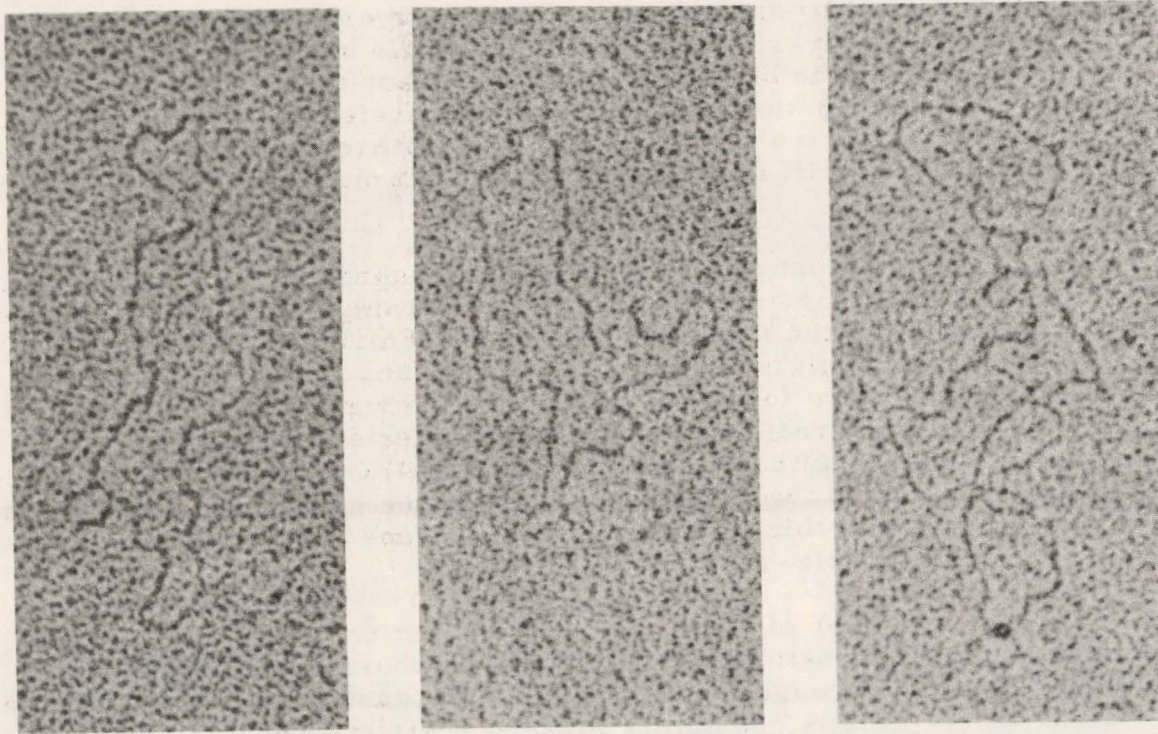
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Fig. 1. Electron micrographs of φX-174 DNA rings.

Fig. 2. Histogram showing length distribution of 186 φX-174 DNA rings. The error shown for the mean is the standard deviation.

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11. We gratefully acknowledge the help of Susan G. Johnson, who took the electron micrographs, and Katherine Le Blanc, Kristin Chung, and John Polacheck, who traced and measured lengths.

INACTIVATION AND RECOVERY OF MICROORGANISMS
AFTER TREATMENT WITH ULTRAVIOLET LIGHT
OR OTHER MUTAGENS[*][†]

Robert H. Haynes[‡]

It is my purpose in this essay to sketch in rather broad outline a
picture of those macromolecular events that appear to be crucial to the
inactivation of bacteria by ultraviolet light (uv). Since the concepts in-
volved are applicable to cell killing by other mutagens such as x rays and
the radiomimetic alkylating agent, nitrogen mustard (HN2), frequent
comparisons will be made to the actions of these agents, and consideration
will also be given to the biological interactions that occur among them.
Although I have necessarily been quite parochial in the actual presentation
of data, most of which come from my laboratory in Chicago, still, my
primary concern has been to discuss general ideas rather than review all
the possibly relevant literature. These restrictions have made my task
the more embarrassingly difficult, since the compelling biochemical
justification for the hypotheses with which my experiments are merely con-
sistent is largely the work of others, and is to be found especially in the
elegant experiments by Richard Setlow and his associates at Oak Ridge.
(For reviews and background material relevant to this article see references
1-7 and the related papers in this Symposium).

The Observation of Photoinactivation

Cell killing, or reproductive death, is the most prominent and most
likely effect resulting from the exposure of bacteria to relatively low in-
cident doses of 2537-Å ultraviolet light. Operationally, it is defined as
failure of the irradiated bacteria to multiply and form visible colonies
(clones) upon incubation on some suitable growth medium. The shape of
the dose-effect curves for uv inactivation (and also for x-ray and HN2
inactivation) is known to depend on many details of the growth, irradiation,
and assay procedures employed.[8-11] Thus, it is convenient to first con-
sider data obtained under some set of "standard" experimental conditions,
and subsequently to try to account for the effects of such modifying factors.

In the experiments described in this paper, the conditions adopted as
standard for the determination of bacterial survival curves were as follows:

the bacteria (mostly strains of E. coli) were grown at their optimal temperature from a small inoculum in a buffered, aerated, complete liquid medium (such as Difco Peptone or Brain-Heart Infusion); the cells were harvested in the stationary phase after 18 to 20 hours of growth, washed, and resuspended (10^6 to 10^8 cells/ml) for irradiation or other experimental treatment in phosphate buffer or distilled water at room temperature; samples of the irradiated suspensions were then plated on Difco Nutrient Agar in order to determine the fraction of cells capable of giving rise to visible colonies after 24 hours' incubation at 37°C.

The experimental features most worthy of note are that the cells were irradiated in stationary phase in a nonnutritive suspension, well below their optimal growth temperature, and then stimulated to multiply by exposure to a complete growth medium. Thus, the fast physicochemical reactions which give rise to the initial photoproducts take place under conditions of minimum metabolic activity, whereas the biochemical events crucial to survival occur slowly during the first few hours after plating. Clearly, the main problem for photobiologists is to determine how these essential biosynthetic reactions are affected by the presence of whatever stable photoproducts have been formed. It is meaningless to ask whether a cell is "alive" or "dead" immediately after irradiation, since operationally, its viability can only be determined by the final outcome of an arbitrary plating assay; and during the lengthy period of growth "lag" that intervenes between the cessation of irradiation and the onset of multiplication, there is ample time for the operation of certain enzymatic processes which can serve to suppress or modify the expression of the initial radiation damage.

It must be recognized that the interpretation of these experiments is complicated by the fact that the physiological state of cells harvested from stationary broth cultures is not well-defined, and that such populations are heterogeneous with respect to macromolecular composition (e. g. , number of nuclei, DNA/protein ratio, etc.), and the time required to attain balanced growth after plating.[12, 13] This situation could be improved by using synchronously dividing cultures,[14] or even log-phase cells grown in a minimal medium. However, the inferences drawn from the data presented below are not substantially compromised by these biological uncertainties. None of the survival curves have resistant "tails" or upward concavities which would arise from relevant population heterogeneities; and the characteristic fluctuations in photosensitivity that have been observed during the growth (in broth or minimal media) of either synchronized[15] or mass cultures[11] of E. coli are not large, and appear primarily as variations in the shoulder, rather than the limiting slope, of the survival curves.

The Concept of Target Macromolecules Relevant to Inactivation

Much of radiobiological thought has been influenced by the idea that cells contain certain nonredundant "targets" or "sensitive sites, " which might be anything from molecules to organelles but whose structural integrity is essential for survival.[16] Damage to an appropriate number of these targets, whether induced by radiation or other agents, is assumed to be lethal, whereas damage produced in other cell components is regarded as irrelevant to inactivation. Although formal target theory provides some useful empirical equations for fitting radiation dose-effect curves, such

exercises are of little heuristic value in advancing our understanding of photoinactivation. However, the problem is still most usefully formulated by assuming the existence of a class of target macromolecules relevant to inactivation, and then inquiring as to their identity, the chemical nature of the changes produced in them by the radiation, and the biochemical consequences of these changes.

At first sight, the number and complexity of the photochemical reactions that can take place in cells exposed to uv seems sufficient to confound any attempt to elucidate the mechanisms of photoinactivation. And furthermore, since it is possible that inhibition of colony formation can arise in several different ways, it might appear whimsical to focus one's attention on any one mode of death. However, in view of the crucial role of DNA in cell growth and division, and the strong uv absorption of the organic bases in nucleic acids, some simplification becomes possible. Two important ideas form the basis of current speculation: (a) uv photoproducts in DNA constitute macromolecular structural defects, which, unless repaired, have a finite probability of giving rise to some biosynthetic failure, such as inhibition of DNA synthesis, or an error in protein synthesis, which leads to cell death; and (b) there are enzyme systems which, under suitable conditions, can serve to repair, eliminate, or bypass certain kinds of structural damage in DNA. Thus, survival is determined by the net probability that radiation-induced structural defects in DNA either persist and prove lethal, or are eliminated through the action of some enzymatic "reactivation" mechanism before the onset of growth on the plates.

This picture enlarges on the traditional target-theoretical description of cell killing in two subtle, but important ways: First, it emphasizes the indeterminancy of death during the immediate postirradiation "repair" period up to the time that failure of clone formation can actually be observed. And secondly, killing is not viewed as the inevitable outcome of the accumulation of any given number of physical hits in sensitive sites, since a substantial fraction of the molecular changes initially produced during irradiation might be subsequently repaired.

In target theory, shoulders on survival curves reflect the departure from a strictly discontinuous threshold response that would arise from statistical fluctuations in the number of physical hits to be found among the cells of an irradiated population at any given level of dose; whereas here, shoulders are to be regarded primarily as a manifestation of the failure of a reactivation mechanism with increasing dose.[7] (This is not to imply that survival curve shoulders cannot also be due to target multiplicity, since Norman has shown[17] that the extrapolated target numbers for uv inactivation of Neurospora conidia can be precisely correlated with the average number of nuclei per conidium).

Since this scheme is based on the assumption that survival after irradiation is determined by the relative probabilities of inactivating and reactivating events, clearly, it is sufficiently general a priori to account for virtually any experimental modification of photosensitivity that might be observed: that is, any apparent increase in sensitivity could be attributed to a suitable combination of increased initial damage or the inhibition of reactivation or both; and vice versa for any apparent decrease in sensitivity.

Therefore, if this picture is to have any predictive value, evidence must be presented, first, to justify the identification of DNA as the most prominent target relevant to photoinactivation, and secondly, to support the idea that uv photoproducts in DNA can be repaired in vivo.

Evidence Implicating DNA as a Target Relevant to Cellular Inactivation

The hypothesis that DNA is the most important macromolecular target, not only for uv, but also for x-ray and HN2 inactivation, is supported, in the most general way, by the fact that all three are mutagenic agents, [2,5,18,19] by their common ability to produce chromosome abberations, [20,21] by the influence of ploidy on cellular sensitivity, [22-24] and by the large ratio of nuclear to cytoplasmic sensitivity observed for a variety of effects in partial cell and microbeam irradiation experiments. [25-29] Uniquely for uv, an historically important observation was the congruence of the action spectrum for the inactivation of various bacteria with the absorption spectrum of nucleic acids; [30-33] and similar action spectra have been obtained for mutations in fungi, [33] mosaicism in maize endosperm, [34] and chromosomal abberations in Tradescantia. [35] Although this indirect evidence is quite comforting, it must be emphasized that many of these effects are at best only remotely related to cellular inactivation, and that the possible importance of damage to protein or to nucleolar or cytoplasmic RNA cannot be rigorously ruled out, even in those cases in which a nucleic acid action spectrum has been observed. [27,36-38] Also, the situation for ionizing radiation is more controversial than for uv. Alper[39] has postulated the existence of non-DNA targets for a substantial fraction of the oxygen-dependent component of x-ray damage; however, this should not be taken to imply that the products arising from the reaction of oxygen with radiation-induced radicals in DNA are irrelevant to inactivation. [40]

The most compelling evidence implicating DNA itself as a target for both uv and x-ray inactivation is to be found in recent work on the sensitization produced by the incorporation of base analogs into DNA. [41] In 1957, Greer and Zamenhof reported[42] that the uv sensitivity of E. coli could be enhanced by the incorporation of 5-bromouracil (5-BU), a halogenated analog of thymine. Since then, both uv and x-ray sensitization by several halogenated pyrimidine analogs, and also purine analogs such as thioguanine, [43] have been studied in considerable detail, not only in bacteria, [43-47] but also in mammalian cells, [48-50] bacteriophages, [51-55] and transforming DNA; [56,57] and Szybalski and Lorkiewicz have shown[57] that the increase in x-ray sensitivity of B. subtilis produced by incorporated 5-BU is paralleled almost exactly by a corresponding increase in sensitivity of the transforming DNA extracted from the same cultures. On the other hand, no sensitization is observed in E. coli grown in the presence of 5-fluorouracil, which -- unlike 5-BU or 5-IU--is incorporated into RNA but not into DNA. [43,46]

In terms of the concepts outlined above, such uv sensitization could be accounted for either on the basis of enhanced killing or impaired reactivating processes associated with 5-BU-substituted DNA, or both. In principle, the former could be due to increased energy absorption (but this is ruled out since the extinction coefficient of 5-BU is actually less than that of thymine at 2600 Å), the greater photochemical lability of 5-BU than of thymine in DNA, [58] or the formation of some new, highly toxic product. However,

current speculation favors a failure of reactivation,[53-55] which could arise in several possible ways, e.g., certain 5-BU photoproducts might be inherently nonrepairable; the prolific yield of photoproducts in 5-BU DNA[58] might surpass the capacity of the system for repair by binding all the available enzyme;[54] or the enzyme might be unable to bind to potentially repairable, non-BU defects because of steric alterations in the secondary structure of DNA caused by neighboring 5-BU residues or their photoproducts in the polynucleotide chain. This last possibility implies that the repair enzymes are not specific for the 5-BU photoproducts themselves and is consistent with the fact that 5-BU also sensitizes to x rays, and that both uv and x-ray sensitization can be achieved by other base analog substitutions,[43] as well as by other agents, such as HN2,[24] ethyl methane sulphonate (EMS),[59] and acriflavine,[60] all of which produce structural alterations in DNA.

Identification of DNA Structural Defects Relevant to Inactivation

Although the chemistry of uv, x-ray, and HN2 attack on DNA has been extensively investigated and many reaction products and secondary structural changes identified,[5, 6, 61-65] so far, only uv-induced thymine dimers have been specifically linked to cellular inactivation and reactivation in vivo.[66] Thymine dimers were first identified in uv-irradiated (2537 Å) frozen thymine solutions by Beukers and Berends,[67] and subsequently found in DNA extracted from irradiated bacteria.[68] Further studies have shown that in the biologically significant dose range they are formed of adjacent thymine residues in the same strand,[69] that dimerization may be reversed by shorter-wave-length radiation near 2400 Å,[70, 71] that they are responsible for up to 70% of the biological inactivation of transforming DNA irradiated at 2800 Å,[72] and that the molecular basis of enzymatic photoreactivation[73] consists solely in the cleavage of thymine dimers.[74, 75] DNA synthesis is inhibited by uv irradiation of E. coli B/r, and this effect can be reversed by exposure to photoreactivating light.[76] Also, it has been shown that thymine dimers constitute a block to DNA synthesis in vitro.[77] Thus, it would appear that the thymine dimer can be regarded as an example of a DNA structural defect which, unless repaired, is likely to inhibit cell multiplication by blocking DNA synthesis.[66]

If the thymine dimerization reaction were uniquely important for photoinactivation, then, other things being equal, those organisms whose DNA is relatively rich in adenine-thymine (AT) base pairs should be more sensitive to uv than those with a low AT content; on this basis, uv sensitivity would be expected to increase approximately as the square of the AT content. Accordingly, we measured, under similar experimental conditions, the dose-response curves for eight species of bacteria whose DNA base compositions span the known range from 28% AT (M. lysodeikticus) to 66% AT (M. pyogenes aureus).[7] Survival curves for these two extreme cases and E. coli B/r (50% AT) are shown in Fig. 1, from which it would appear that a positive correlation exists between uv sensitivity and AT content, although the apparent limiting slope of the survival curves increases at most linearly with AT content. Superficially at least, this would suggest that thymine dimers are important, but not the only uv photoproducts relevant to bacterial inactivation. This is hardly surprising in view of the contribution of cytosine-associated defects to nonphotoreactivable damage,[78] and their role in uv mutagenesis.[79]
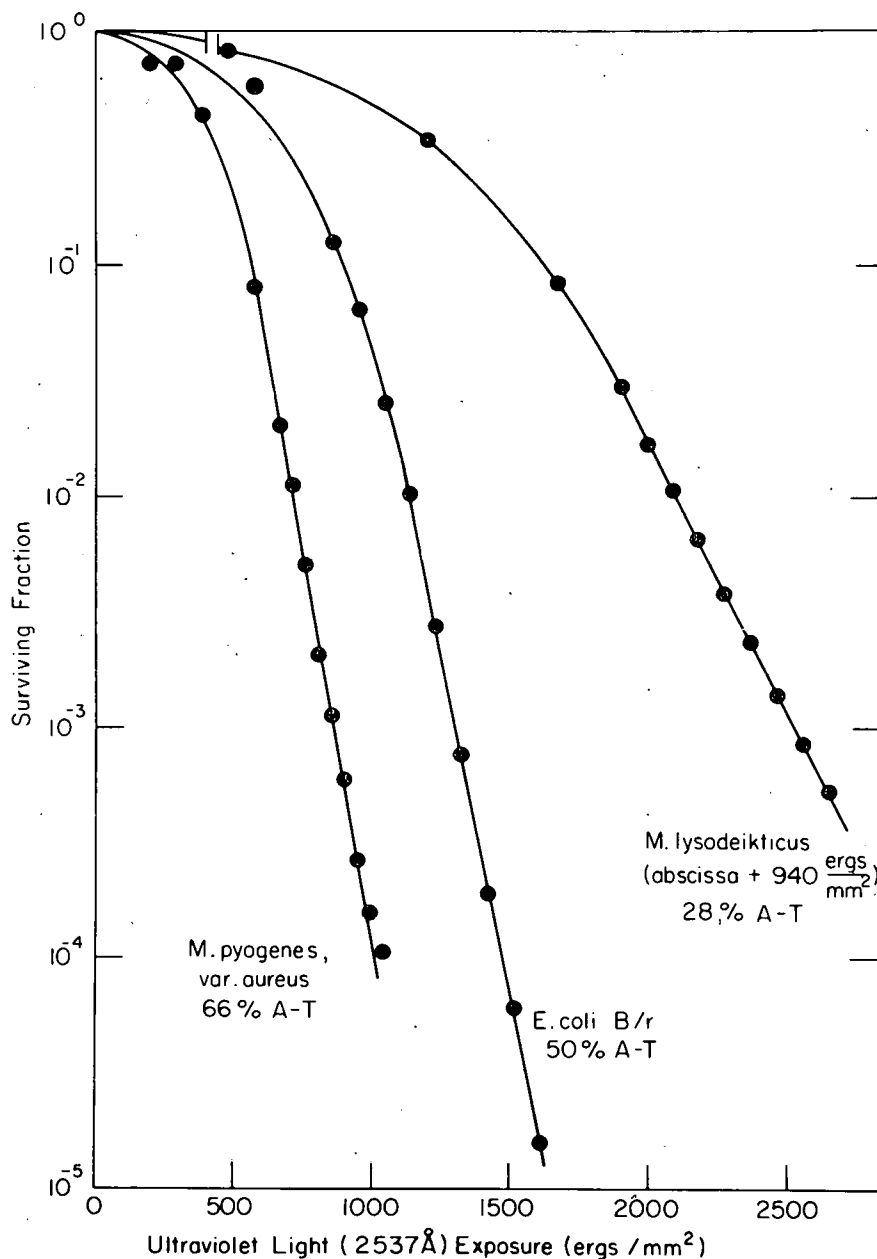
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Fig. 1. Survival curves for the inactivation of three species of
       bacteria of different DNA base compositions by 2537-Å
       ultraviolet light. M. pyogenes aureus and E. coli B/r
       harvested from overnight Brain-Heart Infusion (Difco)
       cultures, M. lysodeikticus from overnight Nutrient
       Broth (Difco) supplemented with 0.85% $NaHCO_3$; cells
       washed twice and resuspended for irradiation in a buffered
       salts solution (pH 7.0), plated on nutrient (salt) agar. DNA
       base compositions given as molar per cent of adenine-
       thymine (AT). Part of the shoulder of the M. lysodeikticus
       curve has been deleted by displacing the abscissa 940 $ergs/mm^2$
   to the left.

Similar base composition correlations have also been observed for x-ray[80] and HN2 inactivation (Fig. 2; Ref. 81), although for each of these agents bacterial sensitivity increases with DNA guanine-cytosine (GC) content. The chemical basis of the x-ray correlation is not clear, since in aqueous solution there exist only small differences in radiochemical lability among the four bases,[61] although when they are irradiated as dry powders, only about one-fifth as much absorbed energy is required to produce a free radical (unpaired spin) in G or C as in A or T[82] [however, this difference seems to vanish when the corresponding nucleotides are irradiated--W. Köhnlein (Yale University), personal communication]. The HN2 correlation is very likely a reflection of the fact that inter- and intrastrand cross linking of guanine residues by alkylation of their N-7 positions is the principal reaction of HN2 with DNA[64, 83] and in this case the magnitude of the correlation is more in keeping with what one would expect on the basis of the quadratic increase of G_pG dinucleotide frequency with GC content.

These correlations of bacterial sensitivity with base composition further support the idea that DNA is an important target for inactivation, and are consistent with chemical data on the possible localization and identity of the relevant defects. However, as will shortly become apparent, the presence and efficiency of reactivation mechanisms are such potent determinants of sensitivity to all three of these agents that it is rather surprising that such correlations can be detected at all.

Intracellular Repair of DNA Structural Defects

The existence of mechanisms capable of reducing the amount of initial radiation damage that is ultimately expressed in irradiated cells has been recognized for some time and a variety of terms have been used to describe them.[84, 85] It is convenient here to distinguish among three such words which have already appeared in this paper, viz., reactivation, recovery, and repair. Reactivation is used to describe intracellular processes which serve to reduce the amount of damage expressed in cells plated immediately after irradiation without any further manipulation or treatment. Recovery is used to denote the enhancement of viability that is often observed if the plating of nonnutritive suspensions of yeast or E. coli is delayed for some hours after irradiation.[86, 87] It is possible, but yet not proven, that the recovery observed upon delayed plating is simply an extension of reactivation, however, it is helpful here to make an operational distinction between the two. Repair is used to denote the actual enzymatic steps that are involved in restoring damaged DNA molecules to a biologically functional form. [It should also be noted that reactivation and recovery occur in the dark, and are to be distinguished from photoreactivation,[73] which may be elicited upon exposure of uv-irradiated cells to visible light (blue) before plating.]

The actual reversal of potentially lethal damage can be most unequivocally demonstrated either in photoreactivation or in dark recovery phenomena, since, until recently, dark-reactivation mechanisms were more inferred than proven. In the yeast Saccharomyces cerevisiae (diploid, SC-6), a great enhancement of viability is observed if nonnutritive suspensions treated with either uv, x rays, or HN2 are stored at 30°C in the dark for 6 or more hours prior to plating.[86] Maximum recovery is usually observed after 24 to 48 hours' storage, and the survival curves obtained upon delayed
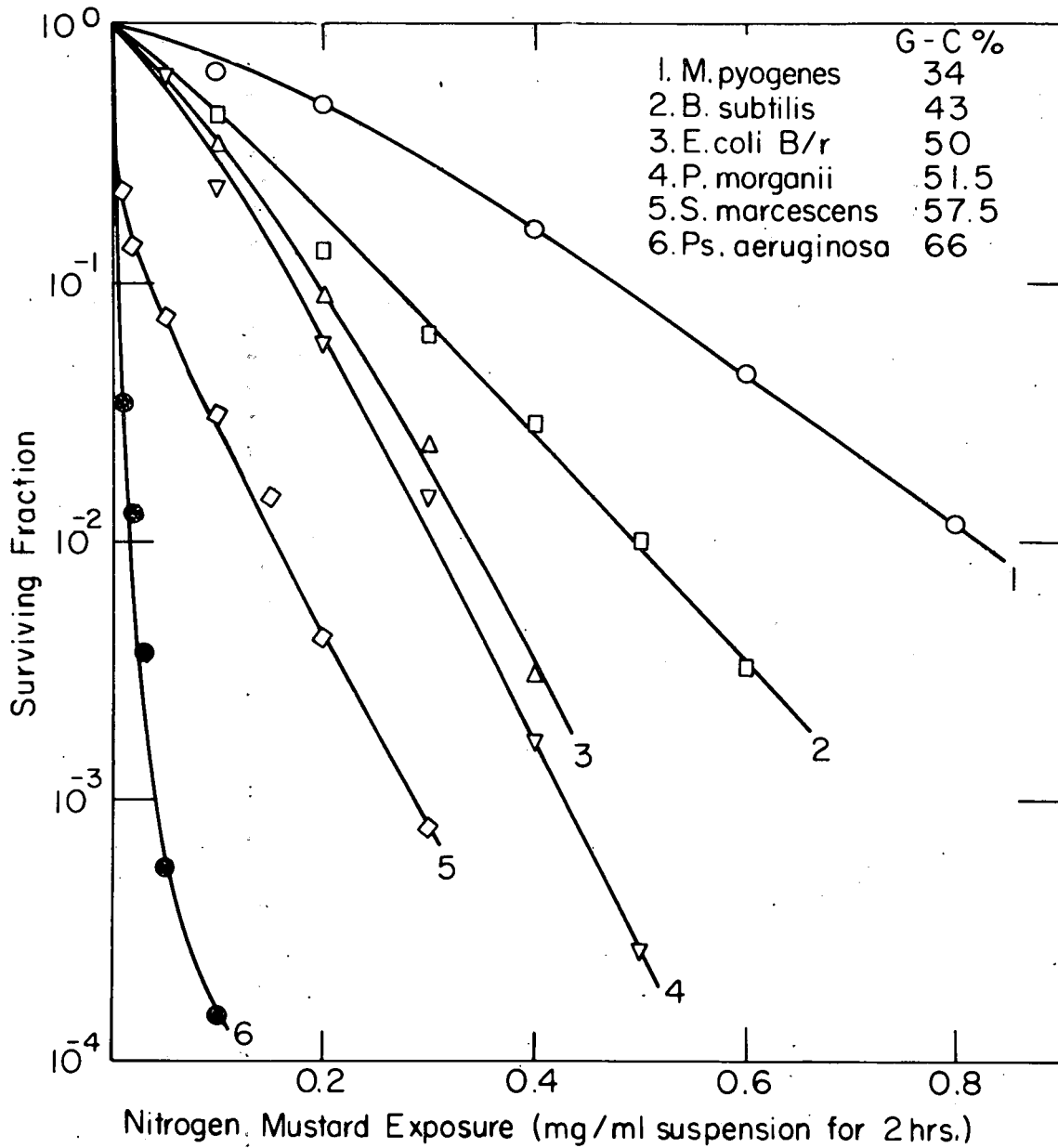
Fig. 2. Survival curves for the inactivation of six species of
bacteria of different DNA base compositions by the bi-
functional alkylating agent, nitrogen mustard. Cells
harvested from overnight Brain-Heart Infusion (Difco)
cultures, washed twice and resuspended in a buffered
salts solution (pH 7.0). Dry, acetone recrystallized HN2
was dissolved in phosphate buffer (pH 7.0) and added im-
mediately to the cell suspensions, which were then in-
cubated in the presence of HN2 for 2 hours at 37°C
before plating. DNA base compositions given as molar
per cent of guanine-cytosine (GC). Note that two species
of similar base compositions (E. coli B/r and P. morganii)
are equally sensitive to HN2 (curves 3 and 4).

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plating are related to those for immediate plating by a constant dose-modifying factor (Figs. 3, 4, and 5); in general, the recovery DMF is greatest for x rays (2.0), intermediate for uv (1.7), and least for HN2 (1.3). No such recovery is observed for either thermal ($60°C$) or photodynamic (acridine orange plus visible light) inactivation. Ploidies higher than diploid also recover, although haploid yeast is unique in that it is incapable of recovery after x irradiation, and displays only slight recovery after uv or HN2 inactivation. The optimal external conditions for recovery are in many ways similar to those for growth, although recovery may be inhibited by the addition of minute amounts of nutrient material to the stored suspension; thus it would appear that the stimulation of growth itself is antagonistic to recovery. The process exhibits temperature and pH optima close to $30°C$ and pH 6.0 respectively; it can be inhibited by the addition of trace amounts of certain cations such as copper, iron, or zinc, and also by metabolic antagonists such as 2, 4-dinitrophenol, potassium cyanide, or sodium azide. Conversely, storage in an atmosphere of pure oxygen or in the presence of ATP (but not AMP) serves to enhance recovery slightly. All these observations are consistent with the idea that dark recovery in yeast is an enzymatic, energy-requiring process, capable of reducing the amount of potentially lethal damage that is expressed in cells treated with inactivating agents known to attack DNA. [59]

A functionally similar reactivation process was proposed by Witkin[38] to account for the repair of those uv-induced DNA defects associated with mutation frequency decline in E. coli B/r. She suggested that this was an enzymatic, energy-requiring process that could act only before the onset of DNA synthesis. Subsequently, the great uv sensitivity of E. coli B_{s-1}[89] relative to that of B/r (Fig. 6) was attributed to the absence of just such a reactivation mechanism. [54, 90] Also, the progeny of uv-sensitive F⁻ strains of E. coli K12 can be made resistant by the incorporation of a genetic locus carried on the male chromosome of uv-resistant Hfr strains, and it was suggested that this locus controls the formation of an enzyme capable of repairing certain uv-induced defects in DNA. [91]

Recently, our understanding of the molecular basis of reactivation in E. coli B/r was notably advanced by the discovery that the dark repair of uv-induced thymine dimers may be initiated by the enzymatic excision of a tri- or tetranucleotide fragment from the damaged DNA strand. [92, 93] It is possible that the resulting gap is enlarged by further nuclease attack and then closed by resynthesis of the excised segment. Although this final re-polymerization step has not been demonstrated in vivo, a suitable reaction has been described in vitro in which the 3'-hydroxyl terminus of the gap would serve as a primer for the insertion of nucleotides in the sequence determined by base-pairing with the opposite intact strand. [94] [N. B.: More recently, D. Pettijohn and P. C. Hanawalt of Stanford University demonstrated a partial degradation and simultaneous synthesis of DNA in uv-irradiated E. coli TAU-bar. Their analysis showed that the newly synthesized DNA consisted of short segments along single DNA strands, and they interpreted this result as being consistent with the idea that certain uv photoproducts in DNA can be repaired by excision and repolymerization with the undamaged DNA strand serving as template (J. Mol. Biol., August, 1964).]
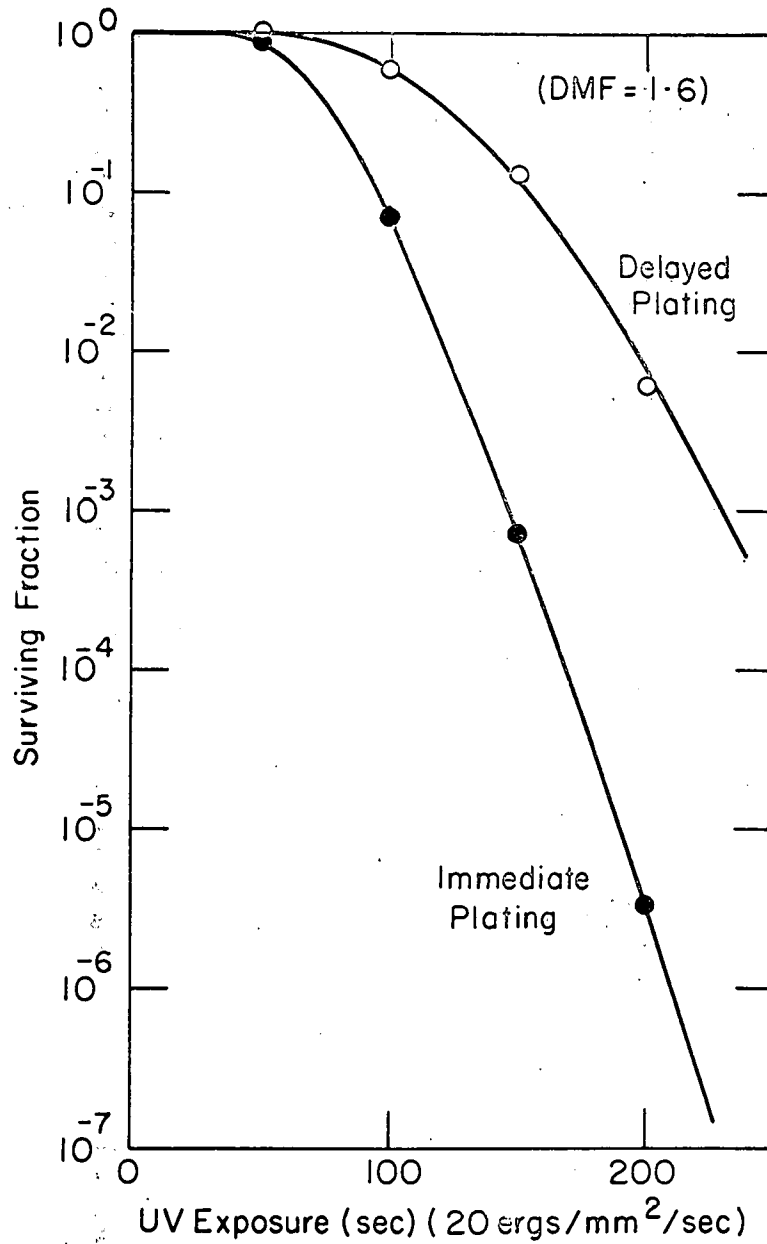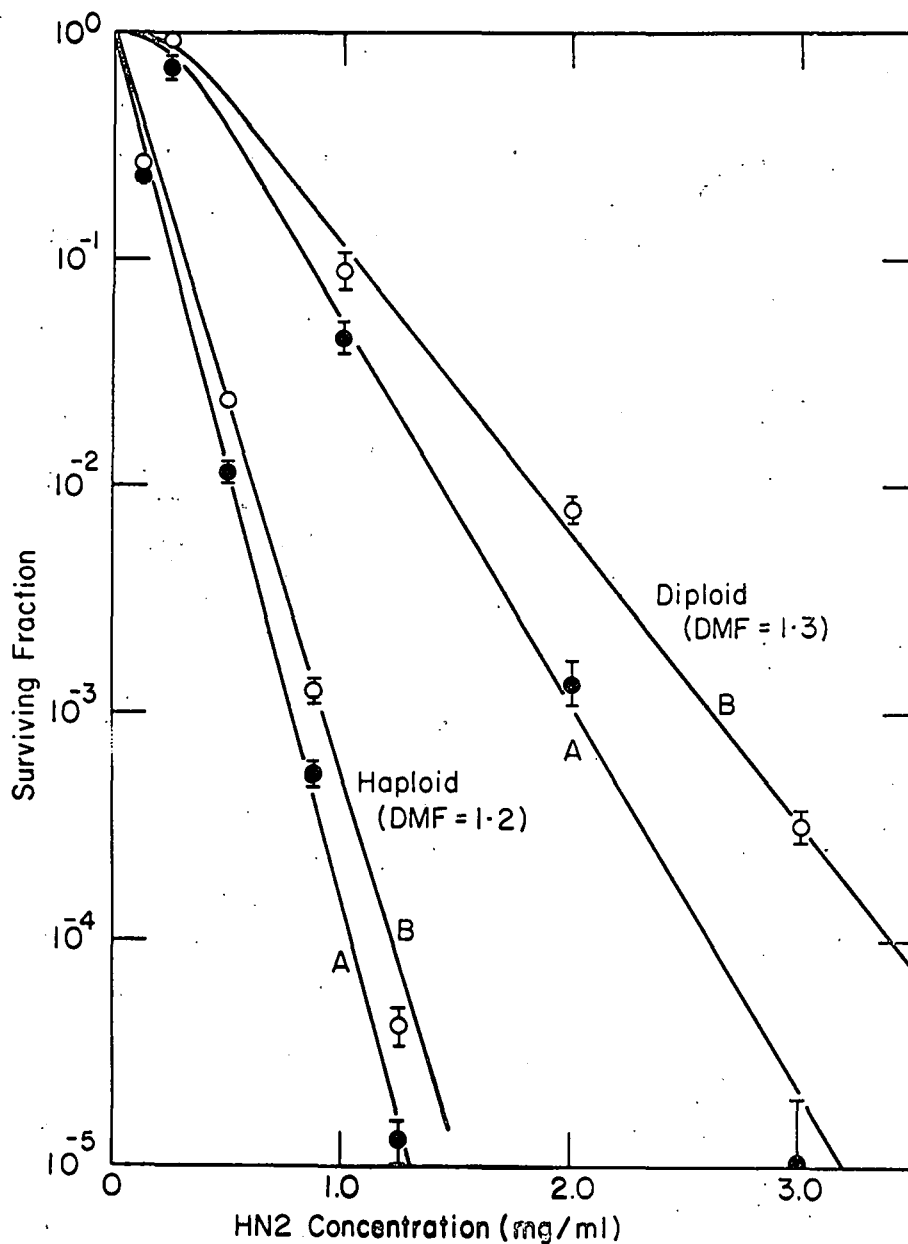
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Fig. 3. Dark recovery of diploid yeast after treatment with
2537-Å ultraviolet light. The cells were harvested
from 2-3-day malt agar (Difco) slants, washed twice,
filtered through Pyrex wool, and resuspended for ir-
radiation in double glass-distilled water (10^6 cells/ml).
Lower curve shows survival in distilled water suspensions
plated immediately after irradiation; upper curve shows
survival in same suspensions after 4 days' storage in the
dark at 30°C.
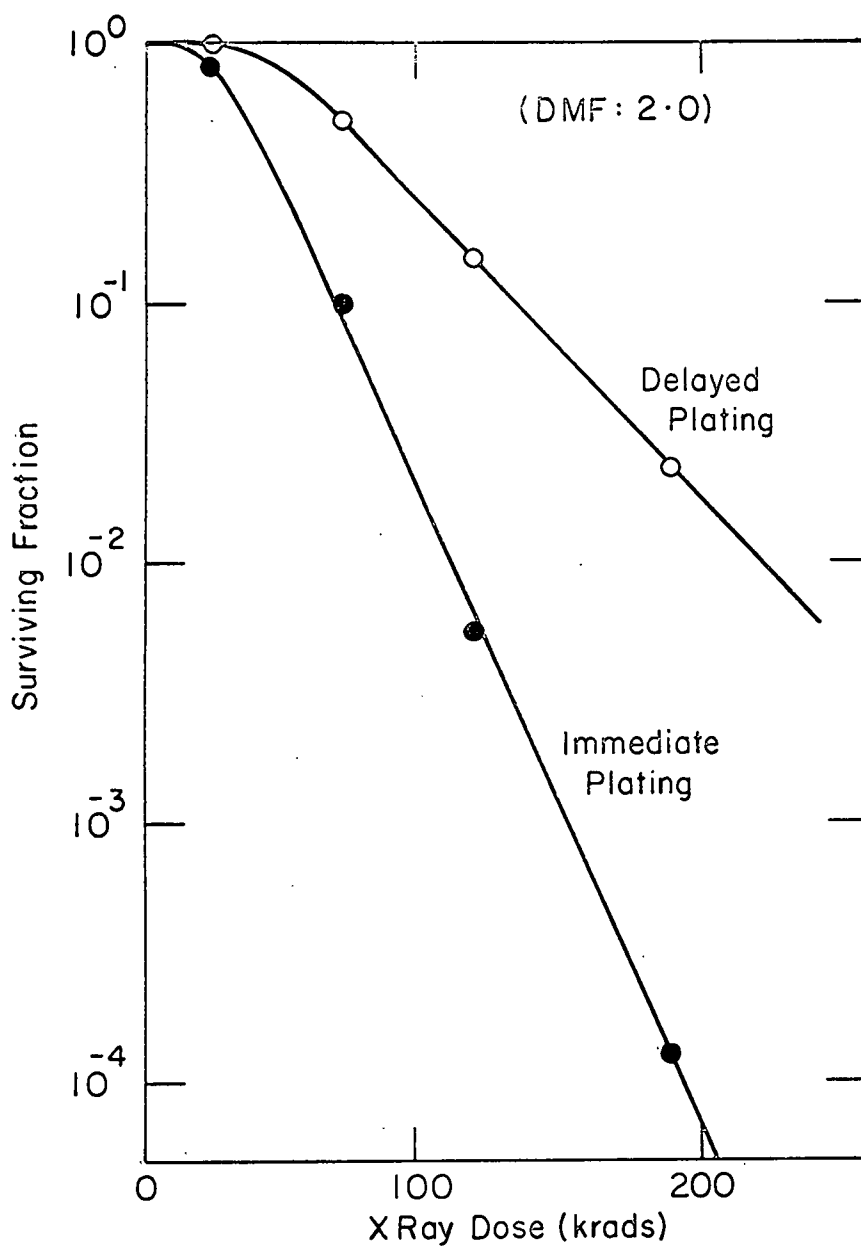
Fig. 4. Dark recovery of diploid yeast after treatment with 150-kvp x rays in the presence of oxygen. Lower curve shows survival in distilled water suspensions plated immediately after irradiation; upper curve shows survival in same suspensions after 4 days' storage in the dark at 30°C.

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Fig. 5. Dark recovery of haploid and diploid yeast after treatment
with nitrogen mustard. The cells were incubated at 30°C for
6 to 8 hours in the presence of HN2; recovery is substantially
independent of whether or not the products of HN2 hydrolysis
are subsequently removed by washing the cells prior to
storage. Curves A show the minimum survival obtained after
addition of the mustard, curves B show the survival obtained
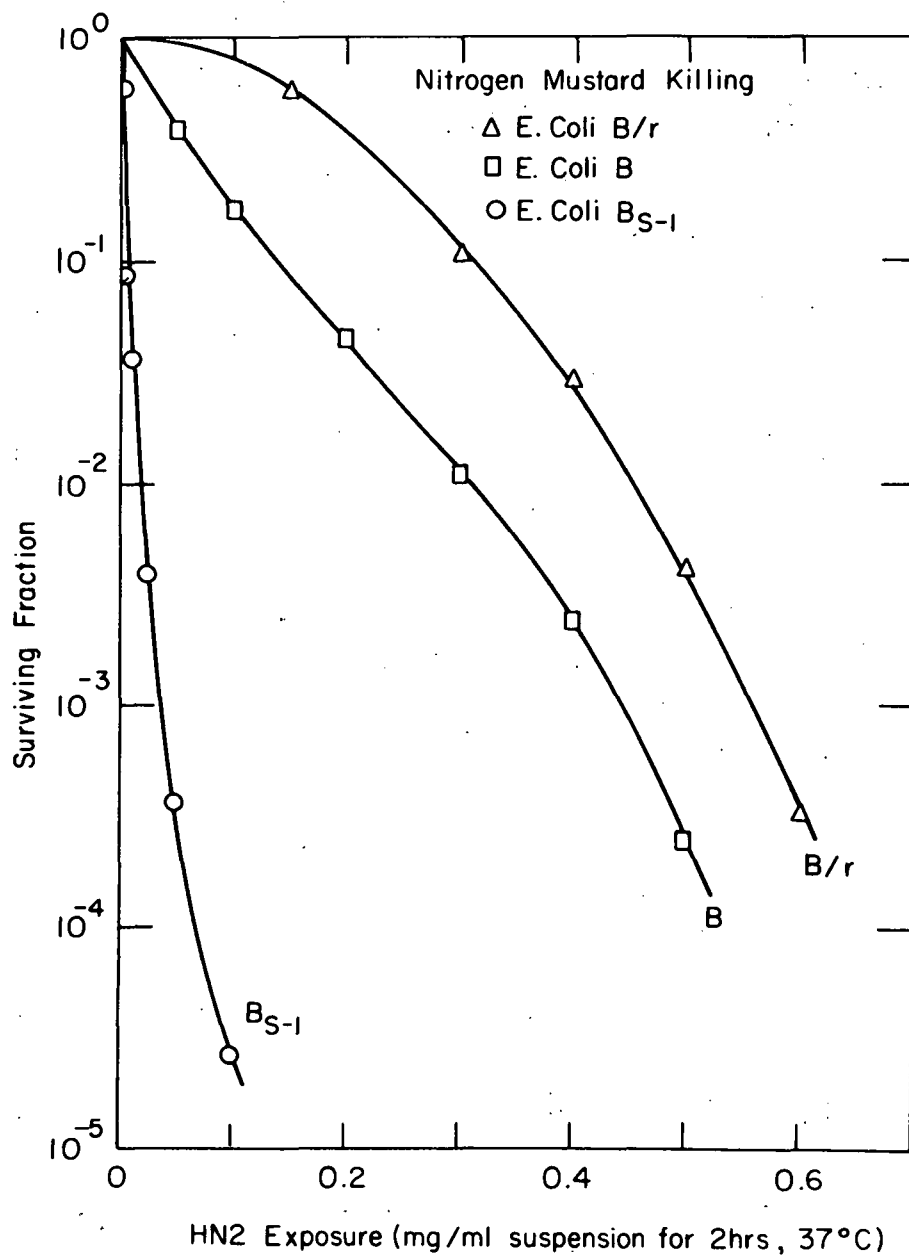after 48 hours' storage in phosphate buffer.
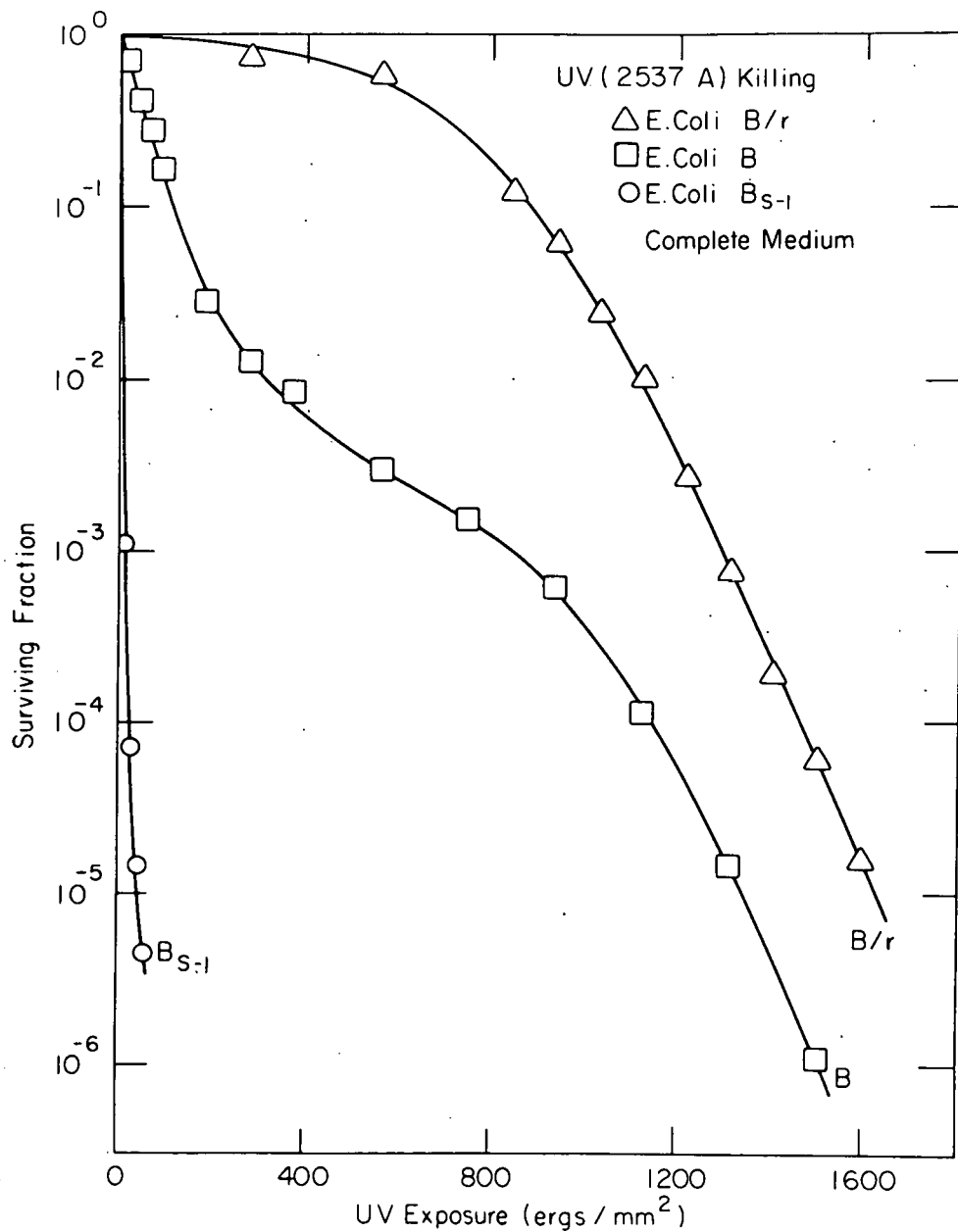
Fig. 6. Comparative dose-effect curves of E. coli B/r, B and
B$_{s-1}$ for inactivation by 2537-Å ultraviolet light. The cells
were harvested from overnight Brain-Heart Infusion (Difco)
cultures, washed twice, and resuspended in a buffered salts
solution (pH 7.0) for treatment with the inactivating agent
and subsequently plated on nutrient (salt) agar plates.

If dark reactivation in B/r is in fact a multistep process initiated by nuclease attack on DNA, it seems reasonable to think that we have here a general "error-correcting" mechanism in which it is not the altered bases themselves that are recognized by the enzymes but rather some associated secondary structural alterations in the phosphodiester backbone. Thus, one might expect that a variety of defects other than thymine dimers can be repaired by this same mechanism. This idea is consistent with the data presented below on the comparative responses of E. coli B/r and the non-reactivating mutant B_{s-1} to inactivation by uv, x rays, and HN2, and also with the patterns of cross-resistance[95] and synergistic interactions that occur among these agents in B/r. [24]

The survival curves for the inactivation of E. coli B/r, B, and B_{s-1} by uv, x rays, HN2, and heat (60°C) under similar growth and incubation conditions are shown in Figs. 6 through 9. It is clear that for each of the three agents known to inactivate mainly through damage to DNA, E. coli B_{s-1} is substantially more sensitive than B/r (Figs. 6-8), whereas all three strains are equally sensitive to heat, which at 60°C probably inactivates through generalized protein denaturation and enzyme inactivation. It should be noted that the uv survival curve for B_{s-1} is exponential (except for a small resistant tail), whereas B/r has a broad shoulder presumably due to the presence of the reactivation mechanism which becomes saturated or fails at sufficiently high doses. However, even in the 5th decade of survival, the apparent limiting slope of the B/r curve is still smaller by almost two orders of magnitude than the initial slope of B_{s-1}, although it is possible that if the B/r curve were extended to higher doses its slope would ultimately become comparable to that of B_{s-1}. The uv curve for the parental B strain has an inflection whose origin is still uncertain, although its limiting slope is similar to that of B/r. In any case, it is obvious from a comparison of the B/r and B_{s-1} curves that the role of parameters such as DNA base composition is vastly overshadowed by that of reactivation mechanisms in controlling photosensitivity.

The survival curves of these three coli strains for HN2 inactivation are strikingly similar in shape to those for uv inactivation (cf. Figs. 6 and 7). However, no thymine dimers are formed by HN2, and since HN2 and uv interact synergistically in B/r, [24] it is likely that the same reactivation mechanism that repairs DNA damage associated with thymine dimers is also capable of repairing HN2-induced defects which cannot be related to thymine dimerization.

Thus, the properties of recovery in yeast and reactivation in E. coli are similar in that both appear to be enzymatic, energy-requiring processes, presumably capable of repairing some fraction of the DNA structural defects induced by uv, x rays, or HN2, but none of the lethal damage produced by heat or acridine-sensitized photodynamic action. [36, 96, 97] The only important difference so far observed is that whereas the dose-modifying factor for yeast recovery after treatment with ionizing radiation appears to be independent of LET (linear energy transfer along the tracks of the ionizing particles), [98] the sensitivity ratio of B/r to B_{s-1} decreases with increasing LET (3.0 for x rays, 1.4 for Po^{210} alpha particles). [96]
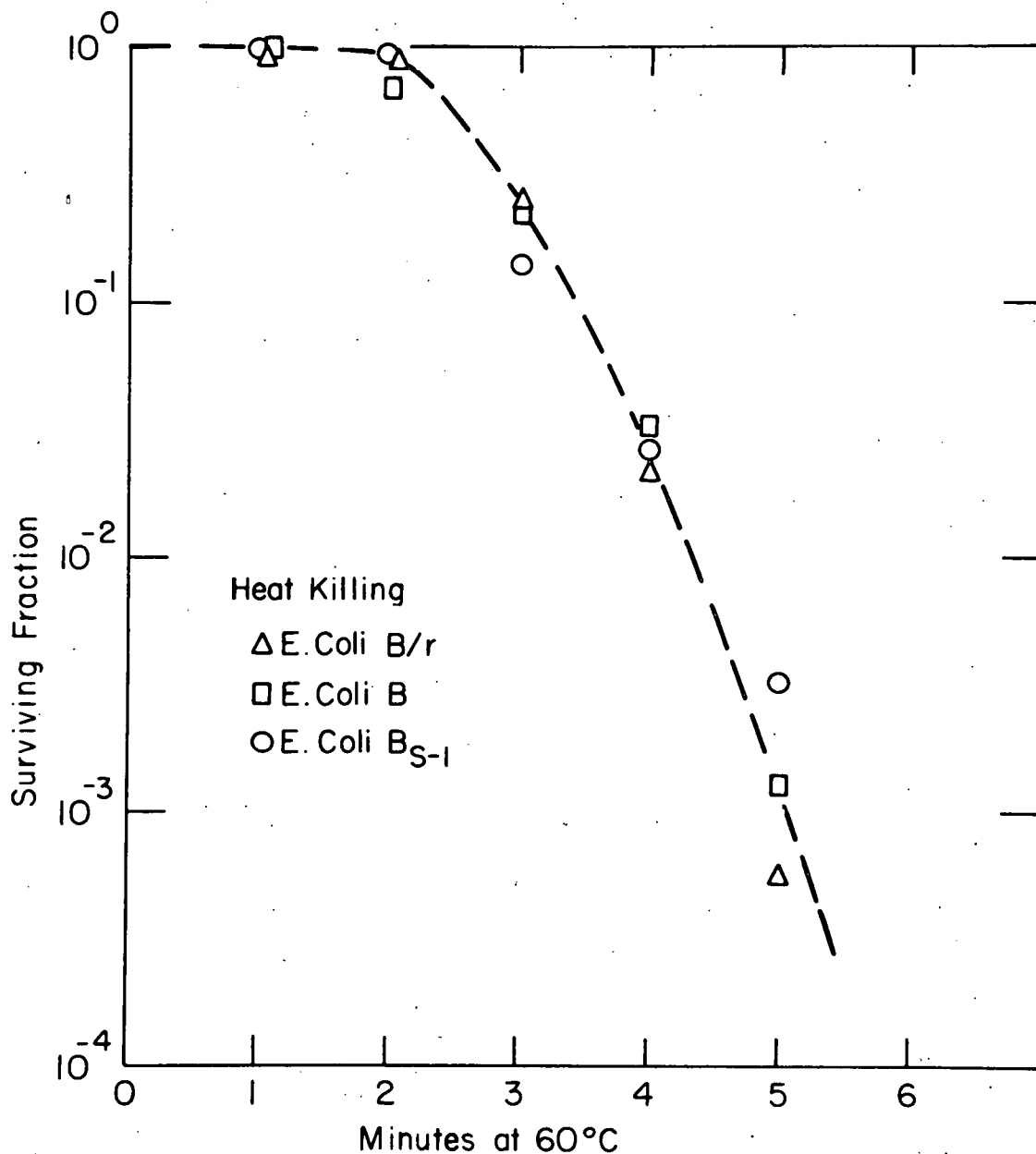
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Fig. 7. Comparative dose-effect curves of E. coli B/r,
B and B$_{s-1}$ for inactivation by nitrogen mustard.
The cells were harvested from overnight Brain-
Heart Infusion (Difco) cultures, washed twice, and
resuspended in a buffered salts solution (pH 7.0) for
treatment with the inactivating agent and subsequently
plated on nutrient (salt) agar plates.
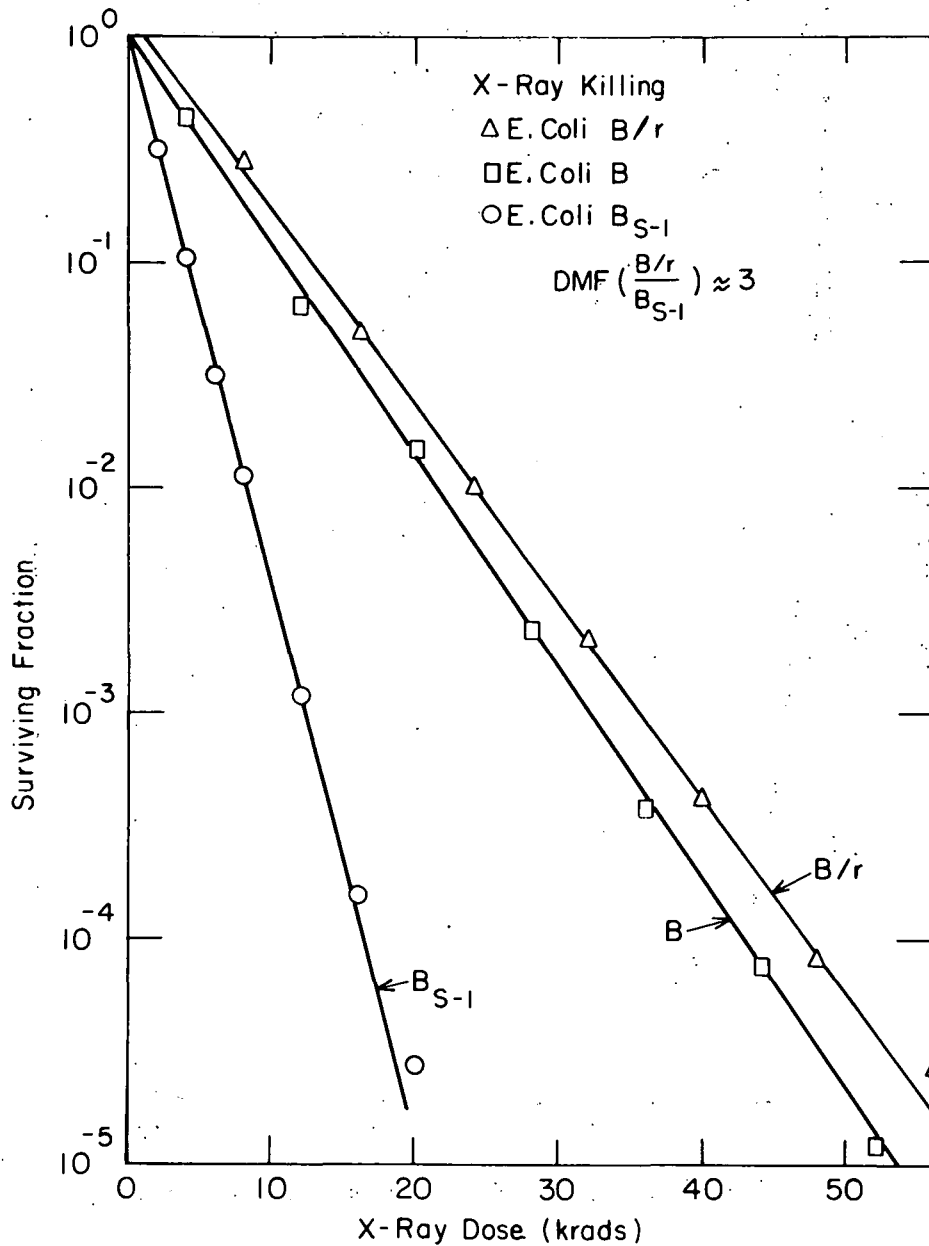
Fig. 8. Comparative dose-effect curves of E. coli B/r,
B and B_{s-1} for inactivation by 150-kvp x rays. The
cells were harvested from overnight Brain-Heart
Infusion (Difco) cultures, washed twice, and re-
suspended in a buffered salts solution (pH 7.0) for
treatment with the inactivating agent and subsequently
plated on nutrient (salt) agar plates.

Fig. 9. Comparative dose-effect curves of E. coli B/r, B and B_{s-1} for inactivation by heat. The cells were harvested from overnight Brain-Heart Infusion (Difco) cultures, washed twice, and resuspended in a buffered salts solution (pH 7.0) for treatment with the inactivating agent and subsequently plated on nutrient (salt) agar plates.

It is not yet known whether or not recovery in yeast is initiated by the excision of defective DNA strand segments. However, recovery may be related to somatic recombination,[24] and in preliminary tests of this idea it has been found that the same storage procedure that elicits recovery in diploid yeast also serves to enhance the frequency of allelic recombination in both unirradiated and irradiated cells.[99] Thus, we have before us the tantalizing possibility that the reactivation and recovery processes relevant to radiation and HN2 damage may constitute a general biochemical mechanism for maining DNA free of certain structural defects, and that at least some of the enzymes involved in recovery are also involved in recombination.

Failure of Reactivation: Sensitization and Synergistic Interactions

Sensitization, meaning simply an enhanced rate of killing with dose, can, in principle, arise either "directly," through some modification of the chemical nature of the initial defects which makes them intrinsically more lethal, or "indirectly," through some failure of the reactivation processes. "Direct" sensitization usually involves the fast reactions leading to the formation of the initial defects, a good example of which is the x-ray oxygen effect, which appears to be based on the reaction of oxygen with radiation-induced free radicals in the target molecules.[40, 100] No comparable sensitization effects have been demonstrated for uv, presumably on account of the difficulty of modifying the very fast excitation processes involved in such reactions as thymine dimerization; however, uv protection resulting from energy dissipation through the sensitized fluorescence of acridine orange bound to DNA has been observed in E. coli.[101]

The properties of the reactivation and recovery mechanisms described above suggest an alternative mode of sensitization based on the inhibition or saturation of these processes. If repair is an enzymatic, energy-requiring process, it should be possible to interfere with it by blocking the energy supply or by suitable attack on either the enzymes or the DNA substrate. For example, recovery in yeast after either x-ray or uv irradiation can be inhibited by suppressing oxidative phosphorylation, by heating the cells for a few minutes at 60°C, or by pretreatment with acridine orange or ethyl methane sulphonate.[59] The effects of heat and EMS are shown in Figs. 10 and 11 respectively: 8 minutes of heating at 60°C, or treatment with 10^{-2} M EMS, reduces the survival of diploid yeast to about 5×10^{-3} (curves D), and in each case the pretreatment completely inhibits recovery after subsequent x-ray or uv irradiation; normal recovery is shown in curves A, and intermediate effects in curves B and C. These experiments suggest that recovery may be suppressed either by thermal inactivation of the repair enzymes (Fig. 10), or by further damage to the DNA substrate (Fig. 12). In addition to suppressing recovery, both of these pretreatments serve to sensitize the cells by reducing the initial shoulders of the survival curves.

The failure of reactivation associated with substrate attack--i. e., the production of additional DNA structural defects--is of interest, since it might provide a common basis for explaining (a) the existence of shoulders on many survival curves, (b) the synergistic interactions that occur among uv, x rays, and HN2, and (c) the sensitizing effects of such mutagens as acridine dyes, alkylating agents, and halogenated-base analogs. If one assumes that the repair of any given defect can be hindered by the existence of
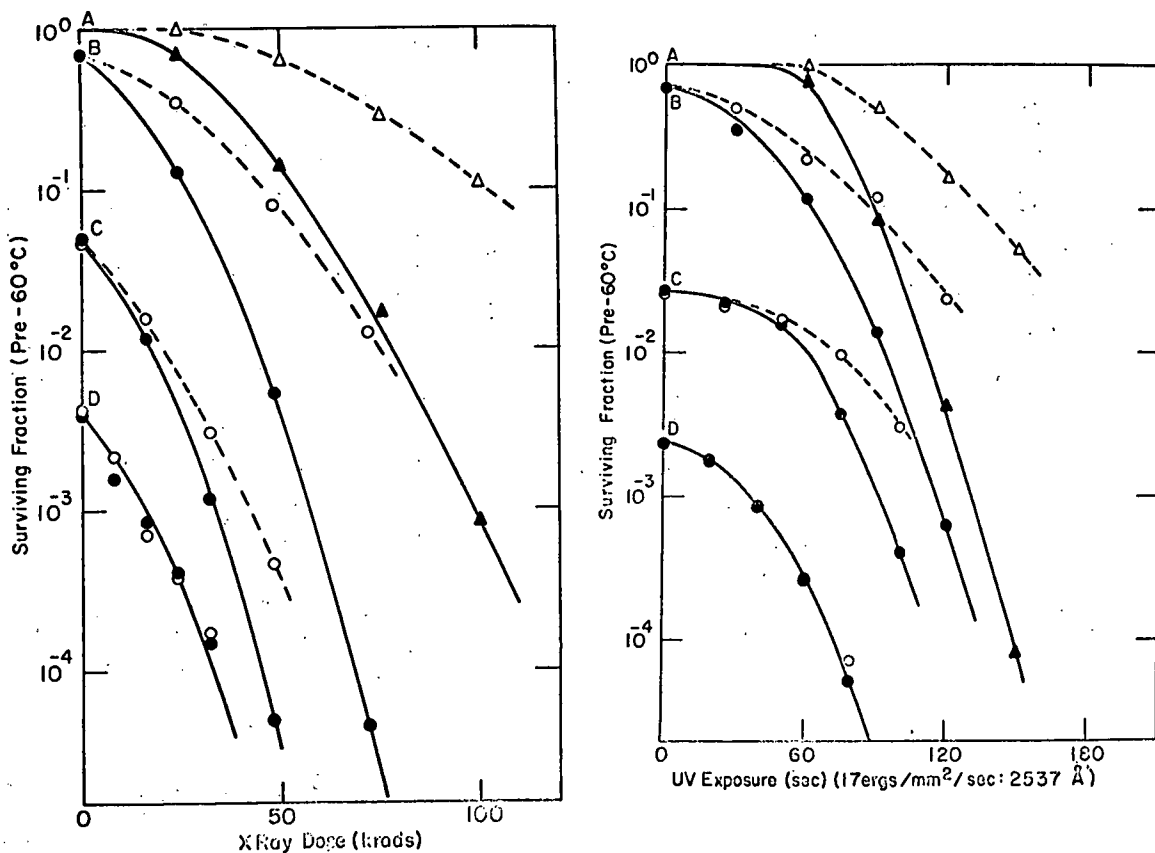
Fig. 10. The effect of preliminary exposure to heat (60°C)
on the recovery of diploid yeast after treatment with
either x rays or 2537-Å uv. (Solid symbols and con-
tinuous curves indicate survival obtained upon im-
mediate plating; open symbols and broken curves
indicate survival obtained if plating is delayed 48 to
72 hours after irradiation.)
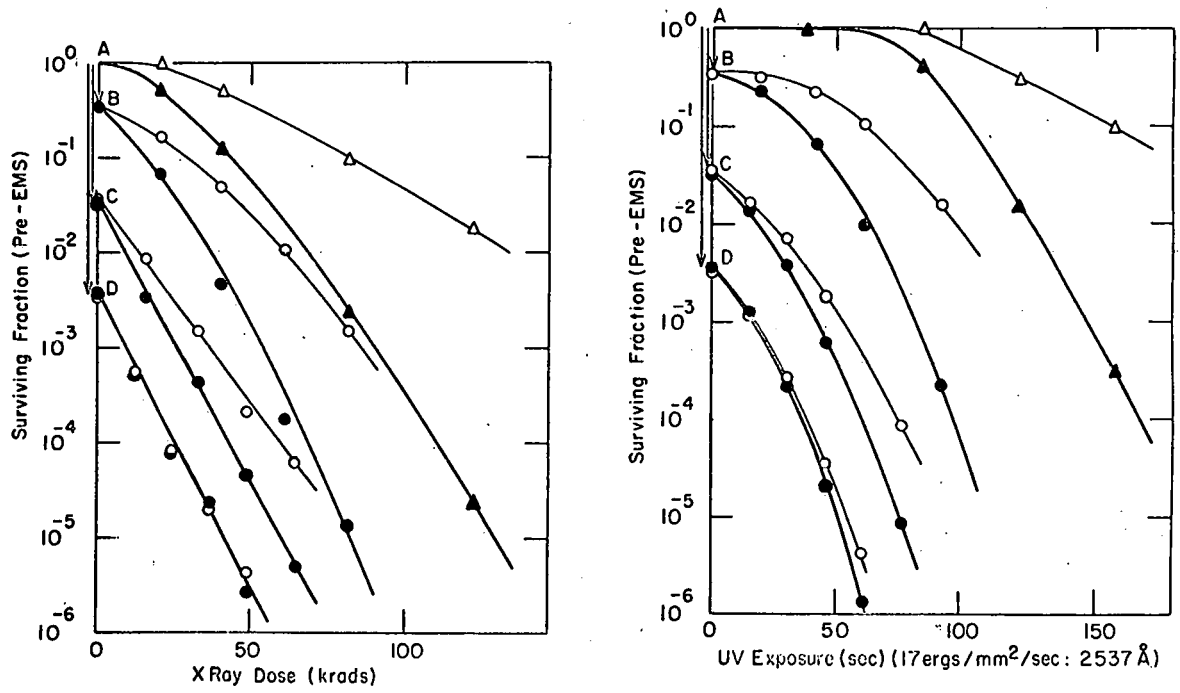
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Fig. 11. The effect of preliminary exposure to 10^{-2} M
ethyl methane sulphonate (EMS) on the recovery of
diploid yeast after treatment with either x rays or
2537-Å uv.   (Solid symbols and heavy curves indicate
survival obtained upon immediate plating; open symbols
and light curves indicate survival obtained if plating
is delayed 48 to 72 hours after irradiation.)

other defects in the polynucleotide chain, it is clear that the net inactivation
probability per defect would be increased in the presence of (a) similar
defects produced by the same agent (hence, shoulders on survival curves--
except in those cases where no reactivation mechanism exists--or the defects
produced up to any practical dose level are so few and sparsely distributed
as to be effectively independent of one another), (b) different defects produced
by other inactivating agents (hence, synergistic interactions), or (c) the
nonlethal defects produced by acridine orange or incorporated base analogs.
Failure of reactivation could arise either from the saturation of the system
owing to the presence of too many potential binding sites (DNA defects) for
the available enzyme supply, [52] or from steric inhibition of binding in the
region of two or more closely spaced defects. Also, it is possible that in
some cases repair could be abortive and actually lead to the creation of
more highly lethal defects, i.e., DNA double-strand breaks might arise
from the attempted excision of defects near the site of preexisting breaks in
the opposite strand.

In addition to accounting for the existence of a shoulder on the uv
survival curve for E. coli B/r and its absence for the nonreactivating
mutant B_{s-1} (Fig. 6), and the inhibition of recovery and shoulder removal
in yeast by treatment with EMS (Fig. 11) or acridine orange, this scheme
can also be used to elucidate the synergistic uv−x-ray, HN2−x-ray and
HN2−uv interactions that occur both in B/r and diploid yeast.[7,24] [No
such interactions have been observed in the nonreactivating mutants of
E. coli, nor do the uv−x or HN2−x interations occur in haploid yeast, which
is unique in its inability to recover after x-irradiation; on the other hand,
haploid yeast is capable of some recovery after uv or HN2 treatment, and
correspondingly there exists an HN2−uv interaction.[24,86]]

The main features of the uv−x-ray interaction in E. coli B/r are
shown in Figs. 12 through 14: First, graded, preliminary uv exposure
increases the x-ray sensitivity up to a factor of 3 over that of control cells
which have not been exposed to uv (Fig. 12); the x-ray sensitivity of E. coli
B_{s-1} is similarly three times greater than that of B/r (Fig. 8). About one-
half of the uv-induced, x-ray-sensitizing damage is photoreactivable. Sec-
ondly, graded, preliminary x-ray exposure partially removes the shoulder
but has no effect on the apparent limiting slope of the uv survival curve
(Fig. 13). And thirdly, the total kill for any given pair of x-ray and uv
exposure is independent of the order in which they are given (Fig. 14),
despite the contrast in the kinetics of the interaction (Figs. 12 and 13). This
third point rules out the possibility that the synergism is based on reactions
between the short-lived products of either radiation, or that any of the
structural defects produced by one are directly "seen" by the other. Rather
it suggests the failure of a postirradiation process, such as a reactivation
mechanism, commonly associated with DNA defects produced by either
radiation. This interpretation is supported by the fact that preliminary uv
irradiation produces a maximum threefold increase in x-ray sensitivity,
which corresponds to the x-ray sensitivity ratio between B/r and B_{s-1}; and
also that the x-ray sensitizing effect of uv saturates out as the uv survival
curve begins its slow approach to a limiting slope (doses ≥ 1200 ergs/mm^2).
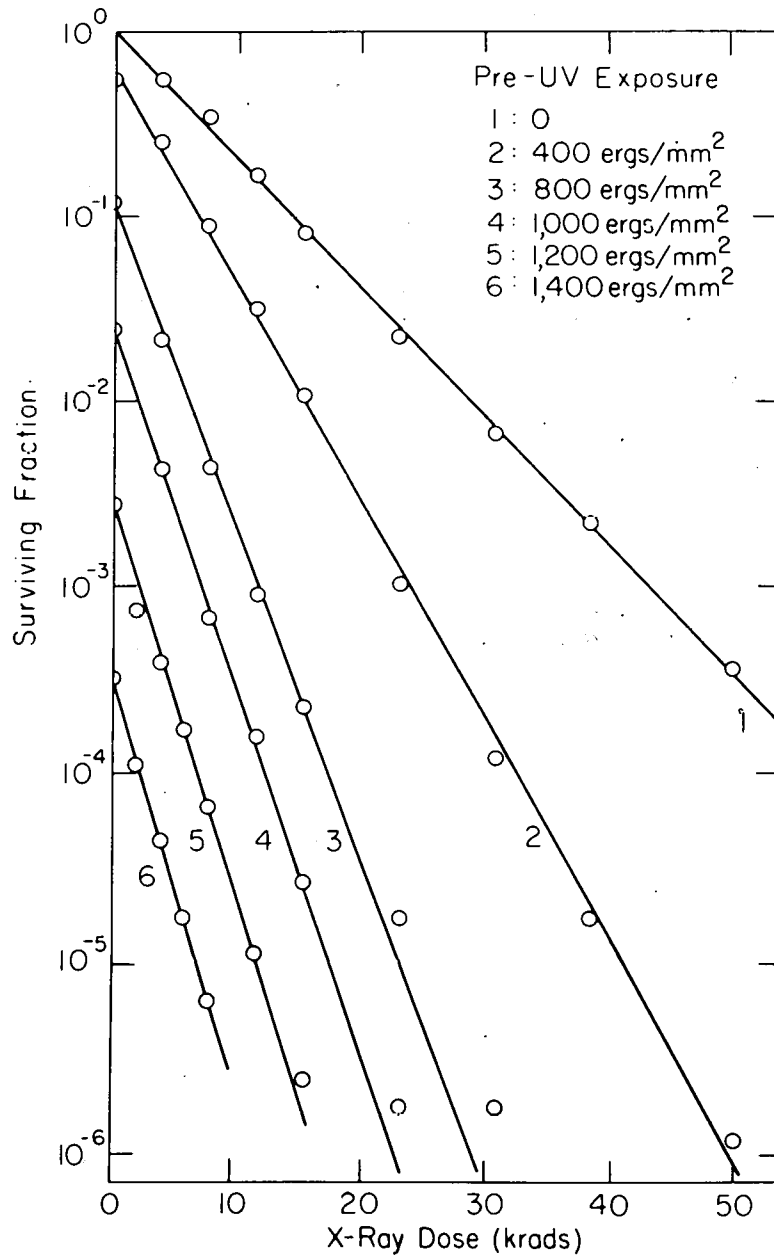
Fig. 12. Synergistic interaction between uv and x rays
in E. coli B/r: preliminary uv exposure increases
the slope of the subsequent x-ray survival curves.
In this experiment the cells were grown in 1% un-
buffered peptone (Difco) and resuspended for irradiation
in distilled water.
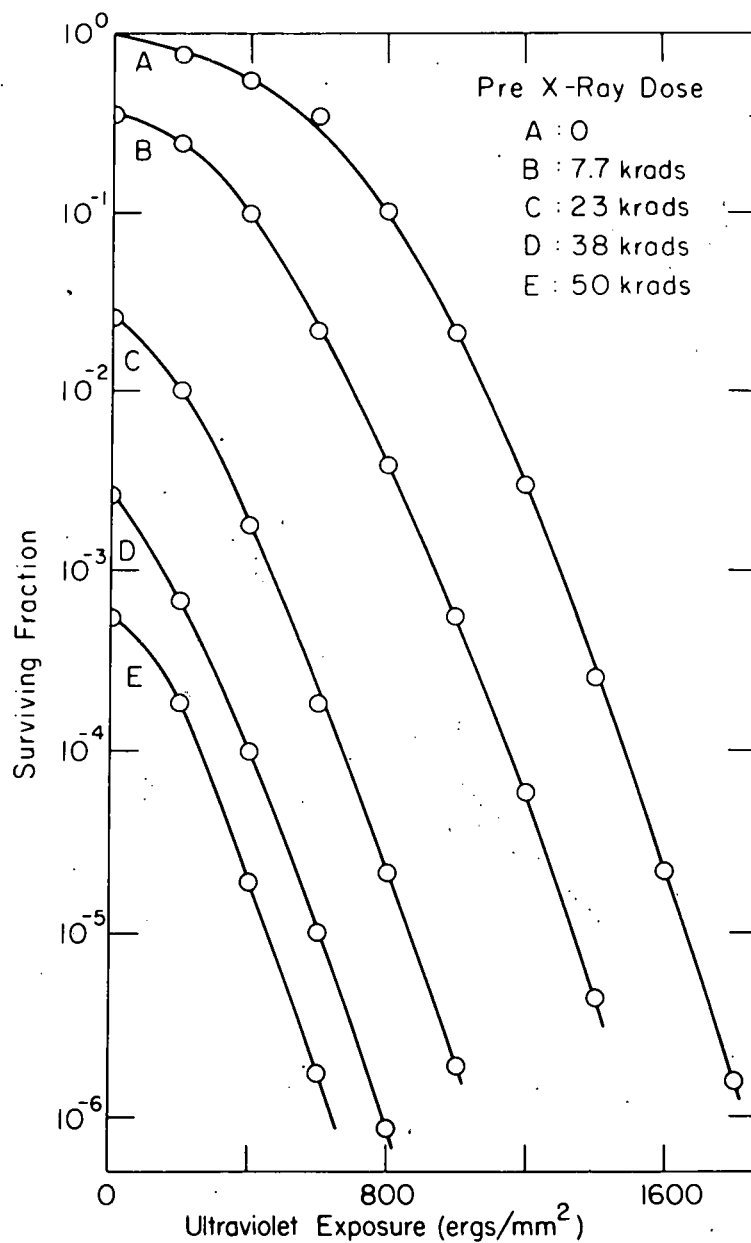
Fig. 13. Synergistic interaction between x rays and uv in
E. coli B/r: preliminary x-ray exposure reduces
the shoulder of the subsequent uv survival curve but
has no effect on its limiting slope.
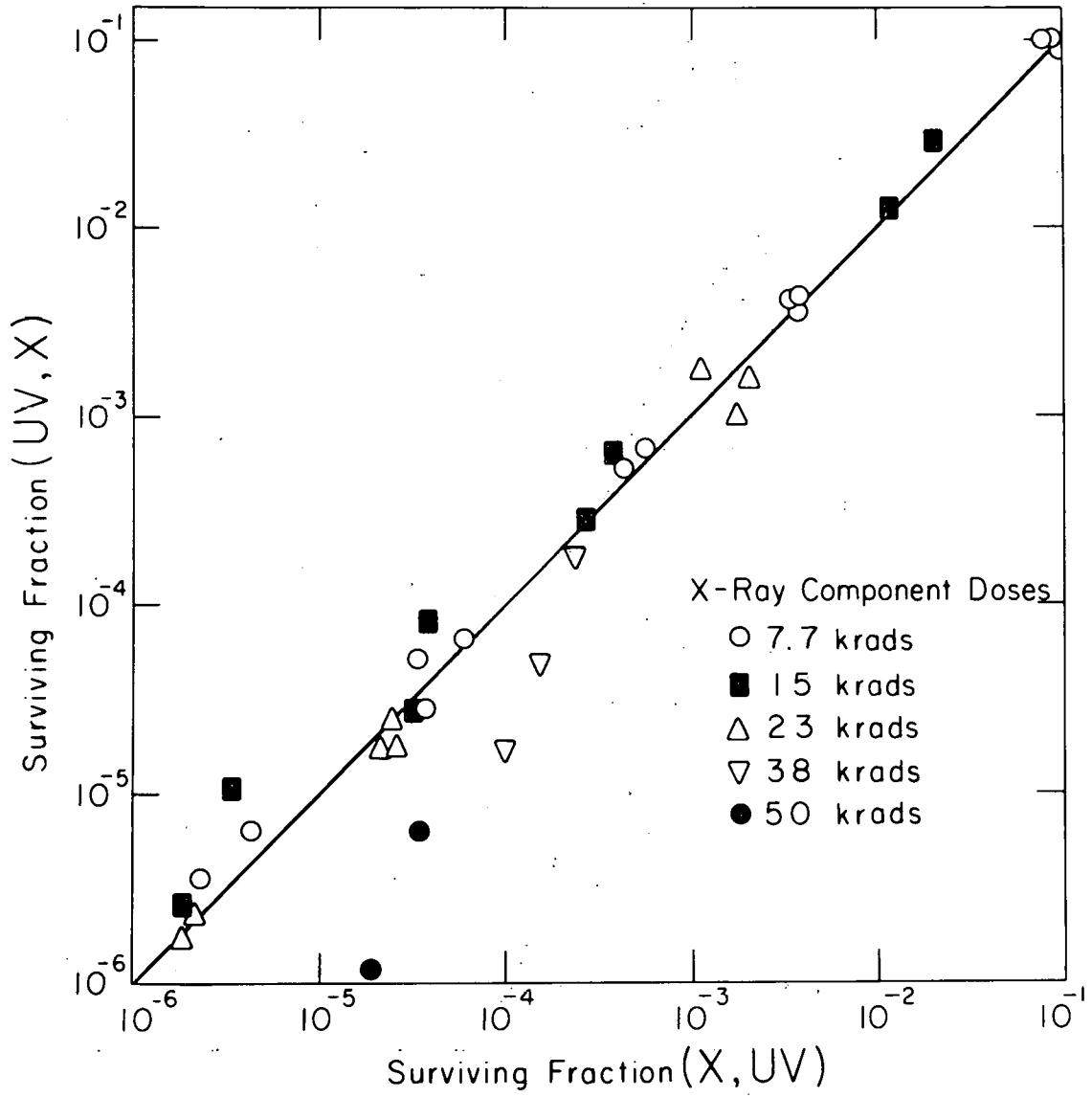
Fig. 14

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Fig. 14. The fraction of cells which survive any combination
of x-ray and uv doses is independent of the order in
which the two irradiations are given. For each point on
this plot the abscissa is the survival level reached by
some ordered pair of doses (x ray, uv), with the x-ray
dose given first; the corresponding ordinate is the
survival level reached by the same pair of doses given
in the reverse order (uv, x ray). The total number of
cells killed is independent of the irradiation order to the
extent that the points fall on the diagonal line drawn through
the origin. Treating the corresponding (x ray, uv) and
(uv, x ray) survival levels as paired data, there is no
statistically significant difference between them (Student's t
ratio 0.74 for 33 degrees of freedom). Further experi-
ments have shown that the apparent departure for the high-
x-ray component points was due to experimental error.

All kinetic features of the interaction can be accounted for if it is
assumed that in the biological dose range the defects produced by x rays are
so sparsely distributed as to be effectively independent of one another (ex-
ponential x ray inactivation), whereas the defects produced by uv become
sufficiently numerous to hinder reactivation (shoulder in uv survival curve):
failure of reactivation due to the presence of uv-induced defects would serve
at most to increase the slope of the x-ray curve up to a factor of 3, com-
parable to that of B_{s-1}; conversely, the presence of x-ray-induced defects
would serve to decrease the shoulder of the uv curve, as the uv dose needed
to saturate the reactivation mechanism would thereby be reduced.

Similar interactions exist in B/r between HN2 and x rays (Fig. 15)
and HN2 and uv,[24] although in these cases it is not feasible technically to
determine whether or not the total kill is precisely independent of the
ordering of the inactivating treatments. Also, it should be noted that the
apparent magnitude of the x-ray sensitization produced by pretreatment with
HN2 is less than that for uv (a factor of 2 for HN2 versus 3 for uv, Fig.
16) which might suggest that relatively few of the HN2-induced defects are
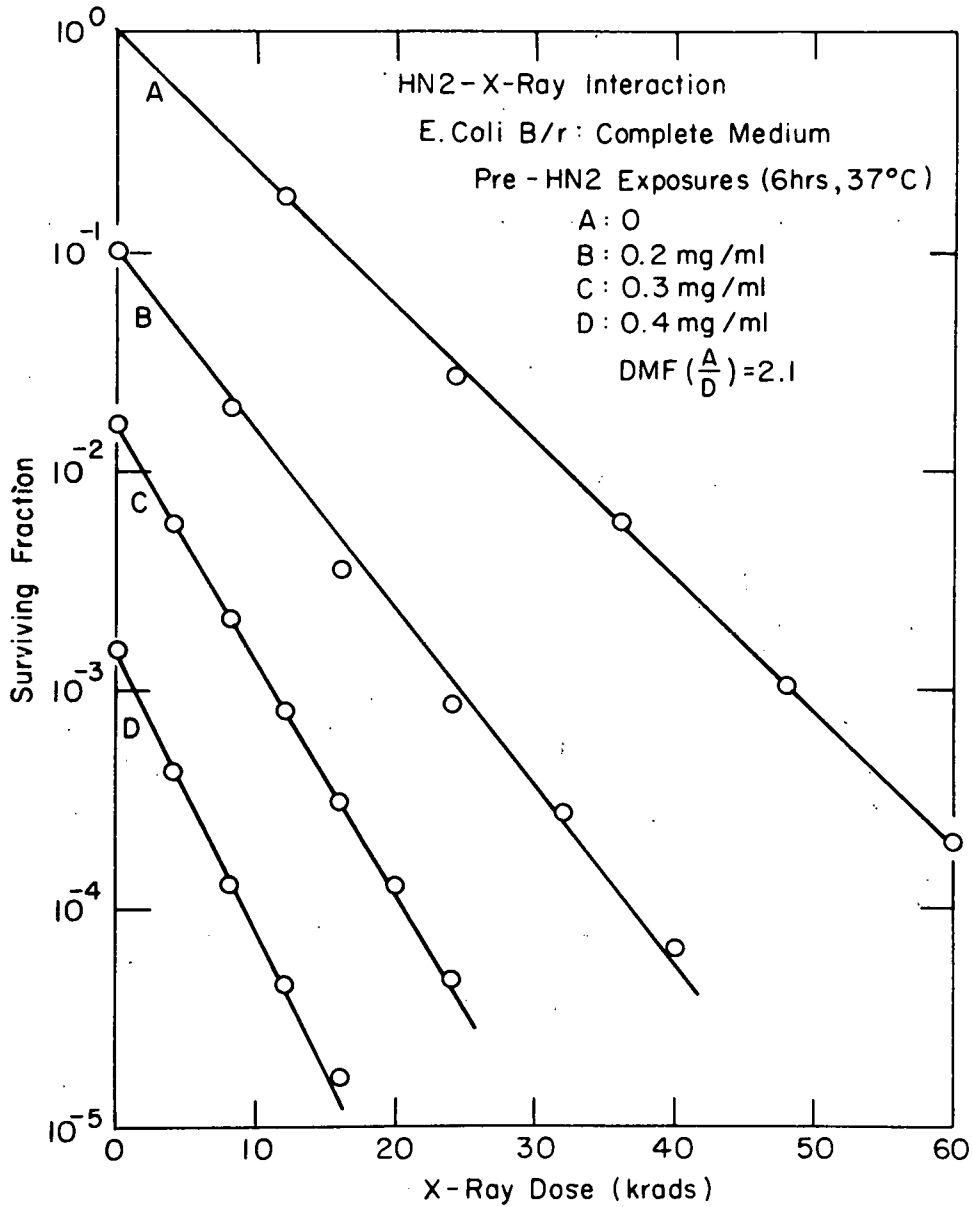susceptible of repair.

Summary

We have described what appears to be the major cause of death in
bacteria treated with ultraviolet light, x rays or nitrogen mustard (HN2):
all these agents produce structural defects in DNA which, unless repaired,
are likely to inhibit DNA synthesis or cause some error in protein synthesis,
which leads to cell death. Thus, survival is determined by the net probability
that these defects either persist and prove lethal, or are repaired after
irradiation during the first few hours of incubation on the plating medium.
The apparent correlations of uv, x-ray, and HN2 sensitivity with DNA base
composition suggest that the relevant damage caused by these agents is
associated with adenine-thymine base pairs for uv and guanine-cytosine
base pairs for x rays and HN2. Intrastrand thymine dimers probably
account for about 50 to 70% of the biological damage caused by uv, and
enzymatic photoreactivation is based upon the cleavage of these dimers.

Both diploid yeast and E. coli B/r possess enzymatic, energy-
requiring reactivation mechanisms capable of repairing DNA structural
defects caused by uv, x rays, or HN2; on the other hand, these repair
processes cannot reverse either thermal ($60°C$) or acridine-sensitized
photodynamic damage, and in fact are inhibited in the presence of acridine
dyes and in cells which have been pretreated either with heat, or with the
monofunctional alkylating agent ethyl methane sulphonate (EMS).

It is suggested that enhanced killing due to the failure of reactivation
in the presence of too numerous or too closely spaced DNA defects may be
a common factor underlying (a) the existence of shoulders on many dose-
response curves, (b) the synergistic interactions among DNA-specific in-
activating agents, and (c) the sensitization produced by substances that
are either incorporated into DNA (halogenated base analogs) or bind to it
(acridine dyes).

HN2-X-Ray Interaction

E.Coli B/r: Complete Medium

Pre - HN2 Exposures (6hrs,37°C)

A: 0
B: 0.2 mg/ml
C: 0.3 mg/ml
D: 0.4 mg/ml

DMF ($\frac{A}{D}$)=2.1

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Fig. 15. Synergistic interaction between HN2 and x rays in E. coli B/r. The cells were incubated in the presence of HN2 for 6 hours at 37°C prior to irradiation. No such x-ray sensitization can be produced by HN2 in haploid yeast.
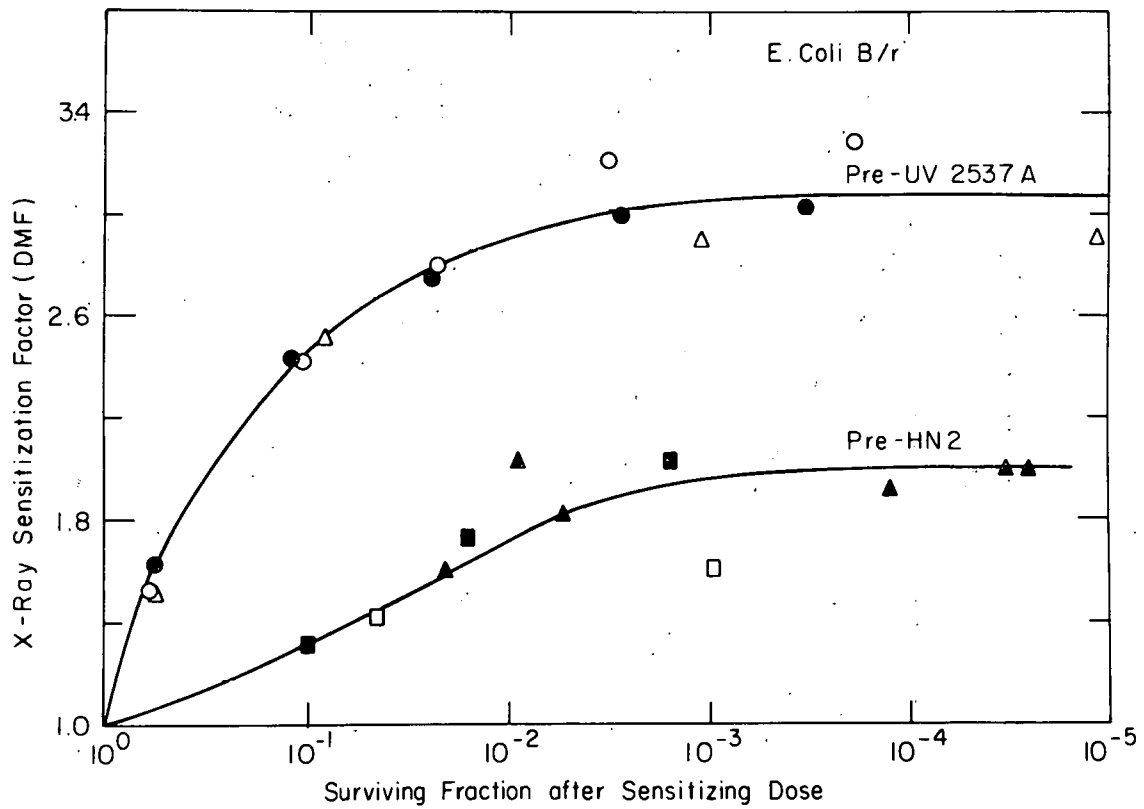
Fig. 16. X-ray sensitization of E. coli B/r produced by
preliminary exposure to uv or HN2, plotted as a
function of the initial survival level reached by the
sensitizing uv or HN2 treatment.

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In view of the existence of all possible synergistic interactions among uv, x rays, and HN2 in diploid yeast and E. coli B/r, the absence of such interactions in those situations where no reactivation or recovery is known to occur, and the similarity in the comparative responses of E. coli B/r, B, and B_{s-1} to inactivation by uv or HN2, it is possible that there exists only one dark reactivation mechanism and that the enzymes involved are specific for DNA secondary structural alterations which may or may not be associated with damaged bases. Such a lack of specificity for the damaged bases themselves appears to be consistent with the recently discovered reactivation mechanism in which repair is initiated by nuclease excision of defective strand segments.

This description of the photoinactivation of bacteria is conceptually simple and broad in scope, although these desirable qualities may reflect more our ignorance than our insight into the complex processes that are involved. However, the study of dark reactivation and recovery phenomena shows promise of rewarding us with a deeper understanding of the temporal continuity of the gene.

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