SENSITIZATION OF PURINE-STARVED BACTERIA TO X RAYS*

By Henry S. Kaplan and F. L. Howsden

DEPARTMENT OF RADIOLOGY, STANFORD UNIVERSITY SCHOOL OF MEDICINE, PALO ALTO, CALIFORNIA

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The susceptibility of bacteria to the lethal effect of ionizing radiation is modified by a number of nutritional and physiologic factors.\(^1\) \(^2\) We have observed an apparently new phenomenon: a striking increase in sensitivity to ionizing radiation in purine-deficient cultures of E. coli, which is reversible by subsequent purine supplementation.

Materials and Methods.—The bacterial strain employed in most of these experiments was E. coli K12, HfrH, sub strain X-662 (pur\(^-\), thiamine\(^-\)), which was kindly provided by Dr. Herbert Marcovich, Service de Radiobiologie et de Cancérologie, Institut Pasteur, Paris, France. This strain has an absolute requirement for a natural purine base; it grows equally well on a mixture of hypoxanthine and xanthine or on adenine.

The organisms were inoculated from agar slants or stationary phase cultures into minimal salts-glucose medium\(^4\) supplemented with thiamine hydrochloride, 10–20 \(\mu g/ml\), and adenine, except where otherwise indicated, at a concentration of 1 \(\mu g/ml\) ("starved") or 100 \(\mu g/ml\) ("supplemented"). They were incubated on a waterbath shaker at 37° overnight, or for shorter intervals as stated. In other experiments, the shift to purine starvation was made during exponential growth; after about 4 hr at 37°, cultures were rapidly filtered and washed on Millipore HA membrane filters, then resuspended and reincubated in prewarmed purine-free medium. Growth was often followed by serial turbidity determinations at 650 \(\mu m\) on a Coleman spectrophotometer, but cell population data in all instances are based upon viable colony counts after plating on yeast-extract agar.

For irradiation, aliquots of the cultures were appropriately diluted to yield 1–3 \(\times 10^7\) cells per ml. An aliquot of this dilution was saved as a zero-dose sample, and the remainder of the dilution distributed in 1.9 ml aliquots to the required number of 35-mm-diameter sterile plastic Petri dishes. The dishes were kept chilled on ice until the time of irradiation. Each dish was individually irradiated in a plastic holder suspended in an irradiation chamber midway between two opposed beryllium window X-ray tubes operating at 50 KVP and 48–50 ma, with 0.3 mm Al added filtration. Under these conditions, the average dose rate in the culture fluid within the dish was 9.9 Krad's per minute.\(^4\) Ultraviolet (UV) irradiation was performed with similarly diluted cultures in plastic Petri dishes on a rotary platform under a low-pressure mercury lamp calibrated to deliver 2,537 Å irradiation at an output of 800 ergs/mm\(^2\)/min at the level of the Petri dish. After irradiation, the samples were appropriately diluted in mineral medium, plated on yeast-extract agar, and incubated overnight at 37°. Colony counts represent the average of 4 plates per dilution per radiation dose. Survival percentages are referred to the average colony count of the unirradiated (zero-dose) sample. In some experiments, the survival of various experimental groups was compared at a fixed radiation dose level of 20 Krad's.

Results.—(1) Enhancement of X-ray sensitivity: Cultures which had been grown overnight on low purine levels (1.25 \(\mu g/ml\)) were strikingly more sensitive to X rays than controls grown with a normal supplement of 100 \(\mu g/ml\) (Fig. 1). The response to X ray was exponential over four decades of killing, reflecting homo-
geneity of radioresponsiveness in the culture population. In several such experiments, the increased sensitivity, as measured by the change in slope of the survival curve, was 2.9- to 3-fold. Radiosensitization by purine starvation has also been noted with three other purine-deficient mutants: H-9 and H-515B, derived from E. coli strain H, which were generously made available by Dr. Joseph Greenberg, Palo Alto Medical Research Foundation, and W-3687, a mutant of E. coli K12, provided by Dr. Esther Lederberg.

In a parallel experiment, purines were supplied at a constant level of 100 μg/ml, while glucose concentration was varied over a 100-fold range (0.008–0.8%). Survival after 20 Krads was clustered within the normal range (20 ± 10%) and showed no consistent trend with decreasing glucose concentration. Thus, it appears that the radiosensitizing effect of purine starvation is not duplicated by starvation for glucose.

(2) Lack of enhancement of UV sensitivity: Purine-starved cultures which exhibited X-ray sensitization showed no difference in survival after UV irradiation, relative to purine-supplemented controls. The effect of purine starvation is therefore selective, producing sensitization to X rays but not to UV.

(3) Effect of degree of purine restriction on radiosensitization: Survival after a fixed X-ray dose (20 Krads) was a function of the purine concentration of the medium in which the cells had been grown and varied approximately 100-fold over the range studied (Table 1).

(4) Relation of incubation time to radiosensitization: Starved and supplemented control cultures were sampled at serial intervals and exposed to a fixed X-ray dose
(20 Krads). Growth curves were reconstructed from the viable count of the zero-dose samples. After a long lag period in both cultures, exponential growth persisted for 7 hr in the control culture, but stopped abruptly at about 3 hr in the purine-starved culture (Fig. 2). Survival after 20 Krads in the purine-restricted culture remained identical with that of the control culture through the lag period and then fell abruptly during the third and fourth hours of incubation to reach a steady low level, which was 50- to 100-fold lower than that of the controls.

When purine-starved cultures were exposed to a graded series of X-ray doses at serial intervals after the start of lag-phase incubation, bimodal dose-response curves were observed, in which the slope of the sensitive component was about 3 times that of the resistant component, and the latter had the same slope (within experimental error) as the unstarved controls (Fig. 3). The absence of intermediate slopes indicates that the radiosensitization due to purine starvation is an all-or-none phenomenon for each individual cell; the graded responses seen in Table 1 are thus a reflection of a progressive change in the relative proportions of sensitized versus resistant cells in the population.

### TABLE 1

<table>
<thead>
<tr>
<th>Purine concentration, mg/ml</th>
<th>Per cent survival</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.036</td>
</tr>
<tr>
<td>2</td>
<td>0.026</td>
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<td>3</td>
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<tr>
<td>20</td>
<td>0.83</td>
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<tr>
<td>50</td>
<td>1.1</td>
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<tr>
<td>100</td>
<td>7.6</td>
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</tbody>
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![Graph showing growth curves and survival after 20 Krads](image-url)
In cultures grown on restricted purine concentrations (0.1–10.0 µg/ml) from the onset of incubation (lag phase) and sampled at serial intervals thereafter for irradiation, the time of transition to partially sensitized, bimodal dose-survival curves was a function of the initial purine concentration. It occurred between the first and second hours of incubation at 0.1 and 1.0 µg/ml (Fig. 4), between the third and fourth hours at 5 µg/ml, and between the fourth and fifth hours at 10 µg/ml. Extrapolation to the ordinate of the resistant components of the dose-response curves of Figure 4 yielded estimates of the resistant cell fraction which decreased exponentially with duration of purine starvation, after a minimum latent period of 1 hr (Fig. 5).

In a second series of experiments, cultures growing exponentially in glucose-salts medium containing 100 µg/ml of adenine were rapidly filtered, washed, resuspended in prewarmed purine-free medium, and reincubated. Bimodal curves were first observed between 15 and 30 min after transfer to the purine-free medium, and a plot of the resistant cell fraction again indicated an exponential transition to the sensitized state, with a latent period of only 5 ± 5 min and a half-time of about 16 min (Fig. 5), in good agreement with the half-time of 18 min for sensitization of the lag-phase cells. Cell replication resumed at a much reduced rate, after an initial period of arrest; there was a 25 per cent increase in viable count at 1 hr, and a 100 per cent increase at 2 hr, relative to the viable count immediately after resuspension in the purine-free medium.
(5) **Reversibility by purine supplementation:** An aliquot of the starved culture of Figure 2 was supplemented with 100 µg/ml of adenine after 5 hr of starvation. The culture was tested for viable count and survival after 20 Krad at 15 min, 1 hr, and 2 hr after the adenine addition. The radiosensitive state proved to be reversible. A small decrease in radiosensitivity was noted as early as 15 min after supplementation, at which time the viable count had not yet changed. However, reversal was not yet complete at the time of termination of the experiment 2 hr later.

In later experiments, cultures supplemented with adenine after 5 and 10 hr of starvation, respectively, and exposed to graded doses of X rays at serial intervals thereafter, again exhibited progressive recovery of normal radioresponsiveness (Fig. 6). Bimodal dose-survival curves containing an appreciable resistant component were first observed 1 hr after supplementation of the 5-hr starved culture, and at 2 hr in the 10-hr starved culture. There was a progressive increase in the resistant component and a corresponding decrease in the sensi-
tized component with time thereafter; by 6–8 hr both cultures had returned to or beyond their normal, prestarvation radiation response. Since the increase in viable count 1 hr after purine supplementation of the 5-hr starved culture was negligible, the radiation-resistant component, which represented over one third of the total cell population at this time, cannot be accounted for on the basis of selective replication of the small residue (1%) of resistant cells in the population. It may therefore be concluded that purine refeeding induces a true reversal of the sensitized state.

Discussion.—It is apparent that purine-requiring mutants of *E. coli* incubated in purine-deficient media suffer a 3-fold increase in sensitivity to the lethal effects of ionizing radiation, as measured by the change in slope of the survival curve. The survival curves are bimodal during the early stages of purine-deficient incubation, reflecting the presence in the culture of a mixture of sensitized and unsensitized cells, the latter having the same sensitivity as control cells. The absence of intermediate slopes indicates that radiosensitization is an all-or-none phenomenon. The conversion of the purineless cell population to the sensitized state follows exponential kinetics, with a half-time of 16–18 min, beginning almost immediately in log-phase cells, and after a minimal latent period of about 1 hr in lag-phase cells. In the presence of small amounts of purine, the onset of sensitization is delayed, the extent of the delay being roughly proportional to the purine concentration in the range 1–10 μg/ml. The sensitized state is completely reversible by the addition of purine, after a variable latent period depending on the duration of prior purine starvation.

The growth of the starved cultures is of interest. Lag phase is not prolonged by purine deprivation and is followed by a period of normal exponential growth, the duration of which is related to purine concentration. Thus, the only apparent effect of purine deprivation is the premature cessation of exponential growth; thereafter, the cultures maintained a quite stable viable count for several hours in the complete absence of purine. This stability is in striking contrast to the rapidly lethal fate suffered by thymine-deficient mutants of *E. coli* grown in the absence of thymine. Recent studies have demonstrated that RNA synthesis during the period of thymine starvation is a prerequisite for "thymineless death." At the moment of purine exhaustion, purine-starved cultures would presumably be forced to discontinue both RNA and DNA synthesis simultaneously, and should therefore be refractory to a purineless counterpart of thymineless death.

Initially, it appeared that the onset of radiosensitization of the purine-deprived cultures was concomitant in time with exponential growth and its premature termination. However, careful comparison of the viable counts of lag- and log-phase starved cultures with the exponential transition of their cell populations to the sensitized state (Fig. 5) makes it clear that a substantial fraction of the cells must become sensitized prior to cell replication. It is equally clear that the restoration of normal radiosensitivity by purine supplementation is not necessarily preceded by cell replication.

That arrest of the starved cultures in some normally radiosensitive phase of the replication cycle is not an adequate explanation for the 3-fold increase in slope observed with purine starvation is clearly brought out by reference to the data of Helmstetter and Uretz. These investigators, working with synchronously dividing cultures of *E. coli*, observed small rhythmic fluctuations in X-ray sensitivity during
the replication cycle, which were entirely attributable to variations in the "shoulders" of the survival curves; no significant variation in the slopes of the survival curves was detected.

The molecular mechanism of purineless radiosensitization remains to be elucidated. However, a considerable body of recent evidence\textsuperscript{9–11} indicates strongly that radiochemical lesions in DNA are largely, and perhaps entirely, responsible for the loss of reproductive integrity in X-irradiated cells. It seems reasonable, therefore, to assume that purine starvation produces a change in the molecular state of cellular DNA which increases its intrinsic radiosensitivity. The postulated change must be consistent with what is now known about the enzymatic mechanism of DNA replication,\textsuperscript{12} and with the experimentally established fact that the phenomenon is reversible. It seems pertinent that the only known instances in which the X-ray sensitivity of a DNA-containing entity, per unit DNA content, is several-fold greater than normal are encountered in the phages \(\phi X174\) and S13, the DNA of which is single-stranded.\textsuperscript{13–16} Semiconservative replication of DNA is thought to require local separation or unraveling of the two strands. It is probable that the region of strand separation is very short, since new deoxyribonucleoside triphosphates are normally available in concentrations adequate to complete the assembly of new strands, and this process presumably occurs at the same rate as, and immediately after, the strand separation step. However, if strand separation and new strand synthesis were to become uncoupled through exhaustion of purine nucleoside triphosphates, continued strand separation would produce DNA molecules containing relatively long regions of enzymatically denatured DNA, each single strand of which might exhibit high intrinsic radiosensitivity similar to that of phage \(\phi X174\) and S13. It would not be unreasonable to expect that the replication of such molecules could resume and go on to completion if additional purine were made available, with restoration of the lesser intrinsic radiosensitivity characteristic of the double-stranded state, which presumably reflects the lesser probability of simultaneous breakage of both strands. One difficulty with this hypothesis which remains unresolved is the fact that phage \(\phi X174\) in the single-stranded DNA form has recently been shown to be several-fold more sensitive than the double-stranded replicative form to UV inactivation,\textsuperscript{17} whereas purine-starved bacteria are sensitive to X rays, but not to UV. However, transforming DNA from \(H. influenzae\) was equally sensitive to UV inactivation in the native and the denatured state.\textsuperscript{18} Perhaps the ring structure of \(\phi X174\) DNA\textsuperscript{19} confers special characteristics on its UV response.

Purine-requiring bacteria grown in the presence of certain analogues of the natural purines also develop increased sensitivity to the lethal effects of X rays.\textsuperscript{20, 21} In view of the known antimetabolite action of these analogues,\textsuperscript{22} consideration must be given to the possibility that their sensitizing effect is at least in part due to purine starvation induced by their competitive interference with the incorporation of natural purines into DNA.

Summary.—Purine-requiring strains of \textit{E. coli} grown on deficient levels of natural purines develop a 3-fold increase in sensitivity to the lethal effects of X rays. There is no concomitant change in UV sensitivity. Exponential growth of the purine-restricted cultures ceases prematurely but is otherwise normal, and viability remains unimpaired for several hours thereafter. The transition from the normal
to the X-ray-sensitized state is an all-or-none phenomenon which follows exponential kinetics, with a half-time of 16–18 min. Cells begin to exhibit sensitization almost immediately in log-phase cultures, and after a minimal latent period of 1 hr in lag-phase. The sensitized state is completely reversible by purine supplementation. Sensitization and its reversal both appear to antedate cell replication. It is postulated that purine exhaustion may arrest the replication of new DNA strands after strand separation has occurred, producing partly denatured DNA molecules of high intrinsic radiosensitivity.

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