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The chemical inhibition of the repair of single-strand breaks in DNA: post-irradiation sensitization to X-rays

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A class of radiosensitizers is discussed which irreversibly inhibits the repair of x-ray-induced single-strand breaks in DNA. Such agents are active when added after irradiation and potentiate x-ray-induced killing in *rec*$^+$ strains of *E. coli* K–12, but have little effect on the viability of x-irradiated *recA* strains which are deficient in their ability to repair x-ray-induced single-strand breaks. Chromatographically-impure samples of hydroxyurea (but not pure samples) have the properties associated with this class of post-irradiation sensitizers.

1. Introduction

It has long been known that the radiation response of bacteria can be modified chemically. Sensitization can be achieved by altering the reactivity of the target molecule, as by incorporating halogenated pyrimidines into DNA (Kaplan 1966, 1967), or by altering the nature of the free radicals produced, as is hypothesized as occurring in the presence of triacetoneamine–N–oxyl (Emmerson 1967). Thiol-binding agents, such as hydroxymercuribenzoate (Bruce and Malchman 1965, Moroson and Tenney 1968 a) are believed to sensitize bacteria by binding to sulphhydryl groups within the cell and preventing free-radical scavenging and chemical repair. A common feature of the sensitizers mentioned above is that they must be present at the time of irradiation.

Another class of sensitizers may be envisaged which act when added *after* radiation damage has been produced. Such agents should function by irreversibly inhibiting the enzymatic repair of the radiation damage. Bacteria genetically deficient in repair processes should show a negligible amount of sensitization by repair inhibitory compounds.

Recent studies have demonstrated that *rec*$^+$ strains of *E. coli* K–12 can repair single-strand breaks induced in DNA by x-irradiation, whereas such breaks are not repaired in *recA* mutants (Morimyo, Zen-Ichiro and Suzuki 1968, Kapp and Smith 1970 a). Since the initial yield of single-strand breaks (for a given dose of x-rays) is the same for the *rec*$^+$ and *recA* strains (Kapp and Smith 1970 a), the increased radiosensitivity of the *recA* mutants is thought to be due to their inability to repair x-ray-induced single-strand breaks.

While studying the effects of DNA synthesis inhibitors on DNA repair, it was found that certain preparations of hydroxyurea (HU) irreversibly inhibited the repair of single-strand breaks. Chromatographically-impure samples of HU, but not pure samples of HU, acted as post-irradiation sensitizers. The active samples of HU were effective when added immediately after irradiation;

they irreversibly prevented the repair of x-ray-induced single-strand breaks in DNA in *rec*$^+$ cells; they greatly reduced the viability of x-irradiated *rec*$^+$ cells; and they produced no detectable sensitization of the irradiated *recA* cells.

2. Experimental

2.1. *Materials and methods*

The derivatives of *E. coli* K–12 used were a *rec*$^+$ strain (AB2497) (Howard-Flanders, Boyce and Theriot 1966) and a *recA*56 mutant (SR74), a thymine-requiring derivative of JC5088 (Clark 1967). Survival curves have shown that the *recA*56 mutant is five times more sensitive to x-rays than is the *rec*$^+$ strain (Kapp and Smith 1970 a). The liquid minimal medium (MM) supplemented when necessary with required amino acids, thymine and thiamine (SMM), and supplemented minimal medium agar (SMM-agar) have been previously described (Ganesan and Smith 1968). All cultures were incubated at 37°c. The HU obtained from Nutritional Biochemicals Corporation was later shown to be chromatographically impure. Other samples (Pierce Chemical Co. and K & K Labs.) were chromatographically pure (Whatman No. 1; solvent: 95 per cent ethanol; spray: ammoniacal silver nitrate) and showed no radiation potentiating activity†. Stock solutions of 1 M HU were prepared immediately before being used. Irradiations were performed with a 50 kVp x-ray unit (Loevinger and Huisman 1965) at a mean dose-rate of 8·8 krads/min.

2.2. *Alkaline sucrose-gradient studies*

To study the effect of HU on the repair of x-ray-induced single-strand breaks, *rec*$^+$ cells in exponential growth were labelled with ^{3}H-thymine (17·4 Ci/mmole, New England Nuclear; 25 μCi/ml. medium) for 3 to 4 hours, harvested at 1 to 5×10^7 cells/ml., suspended in MM without glucose and irradiated at room temperature in air. The cells were then reincubated in SMM containing 0·1 M HU for various times, washed twice in 0·05 M Tris (pH 8·1) and transformed into spheroplasts (Rupp and Howard-Flanders 1968). Samples (20 μl.) of spheroplasts (equivalent to $\sim 10^6$ cells) were lysed and sedimented in alkaline-sucrose gradients as described elsewhere (McGrath and Williams 1966, Kapp and Smith 1970 a).

Figure 1 demonstrates that reincubation in SMM containing 0·1 M HU (impure) for periods of 30 and 60 min immediately after irradiation inhibits the repair of single-chain breaks. In contrast, the irradiated cells reincubated in SMM in the absence of HU show a rapid return of the sedimentation profile to that of unirradiated cells, indicating the rejoining of single-strand breaks.

To investigate the reversibility of the HU-induced blockage of repair of single-strand breaks, prelabelled *rec*$^+$ cells in exponential growth were irradiated and reincubated in SMM containing 0·1 M HU for 60 min. The HU was removed by filtration and the cells were reincubated for an additional 60 min in SMM. The samples were then sedimented in alkaline-sucrose gradients.

† While preparing this manuscript the work of Moroson and Tenney (1968 b) was called to our attention. These authors found no sensitization of *E. coli* B/r when the cells were x-irradiated in the presence of HU (purity and manufacturer not specified) in oxygen, but did find sensitization when they were irradiated in the presence of HU in the absence of oxygen.

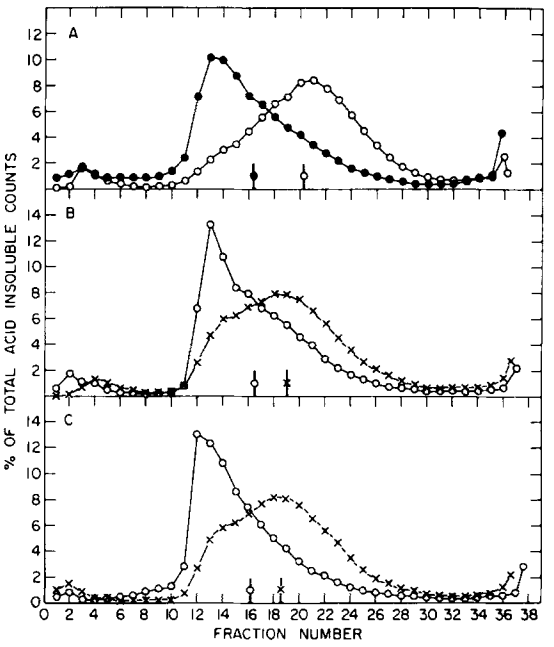

Figure 1. Effect of chromatographically-impure HU (Nutritional Biochemicals Corp.) on the repair of single-strand breaks in *E. coli* K–12 *rec*$^+$ cells. Cells in exponential growth labelled with ^{3}H–thymine were irradiated, converted to spheroplasts and lysed on top of alkaline-sucrose gradients (5–20 per cent sucrose in 0·1 N NaOH). Sedimentation was performed in a SW 50·1 rotor for 105 min at 30 000 r.p.m. at 20°c. The direction of sedimentation is from right to left. The short vertical bars under the peaks indicate the first moments of the distribution of radioactivity (Kaplan 1966). Less than 50 per cent of the DNA was degraded during the 60 min of re-incubation, and HU treatment had no effect on the amount of degradation. (A) No incubation: ●, unirradiated, ○, 22 krads; (B) incubation for 30 min in SMM: ○, 22 krads, no HU; x, 22 krads, 0·1 M HU; (C) incubation for 60 min: ○, 22 krads, no HU; x, 22 krads, 0·1 M HU.

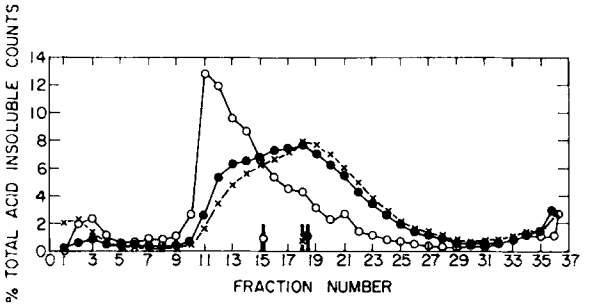

Figure 2. Lack of repair of single-chain breaks in x-irradiated *E. coli* K–12 *rec*$^+$ cells during incubation in impure HU (Nutritional Biochemicals Corp.) or after removal of HU. Lysis and sedimentation were performed as in figure 1. ○, 22 krads, incubated 60 min in SMM without HU; x, 22 krads incubated 60 min in SMM containing 0·1 M HU; ●, 22 krads incubated 60 min in SMM containing 0·1 M HU and then for an additional 60 min in SMM without HU.

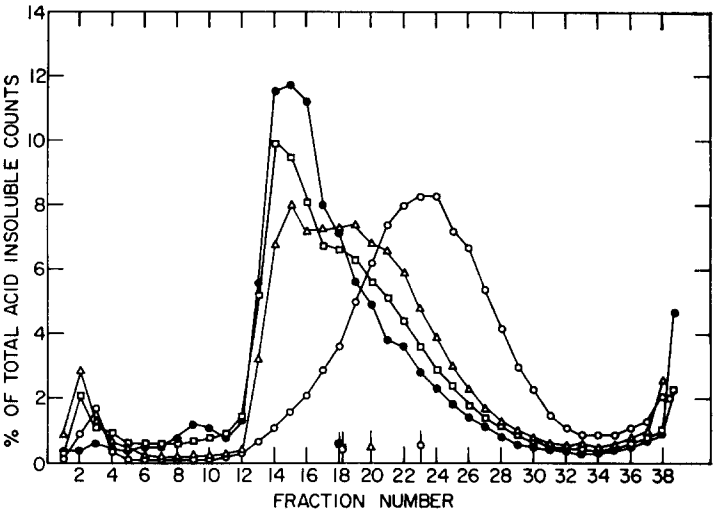

Figure 3. Reversal of the partial inhibition of repair of x-ray-induced single-chain breaks in *E. coli* K–12 *rec*$^+$ cells after the removal of chromatographically-pure HU (Pierce Chemical Company). Lysis and sedimentation were performed as in figure 1. ●, unirradiated cells, no incubation; ○, 22 krads, no incubation; △, 22 krads, incubated 60 min in SMM containing 0·1 M HU; □, 22 krads, incubated for 60 min in SMM containing 0·1 M HU and then washed and incubated for 60 min in SMM without HU.

The rejoining of single-strand breaks was inhibited during the 60-min incubation in the presence of impure HU and continued to be inhibited after the removal of the HU and subsequent reincubation in SMM for 60 min (figure 2). Thus the chromatographically impure samples of HU irreversibly inhibit the repair of x-ray-induced single-strand breaks.

In contrast to these findings, a chromatographically-pure sample of HU (Pierce Chemical Company) produced only a partial blockage of repair of single-strand breaks, which was reversed upon removal of the HU (figure 3).

2.3. *Viability studies*

Since the repair of x-ray-induced single-chain breaks is important for the survival of irradiated *rec*$^+$ cells (Kapp and Smith 1970 a), treatment of irradiated cells with an agent that inhibits the repair of single-chain breaks should sensitize *rec*$^+$ cells to x-ray-induced killing, but should have little effect on *recA* mutants, which lack the ability to repair single-strand breaks. To test this hypothesis, irradiated cells were treated with HU for various periods of time before plating for viability. Figure 4 shows that impure HU markedly potentiates the killing of x-irradiated *rec*$^+$ cells.

The results of similar studies on the *recA*56 mutant (figure 5) are in marked contrast to those for the *rec*$^+$ strain. There appears to be no potentiation of killing of x-irradiated *recA*56 cells by HU at either dose of radiation employed.

Chromatographically-pure samples of HU (Pierce Chemical Co. and K & K Labs.) were also tested for their ability to potentiate x-ray-induced killing in

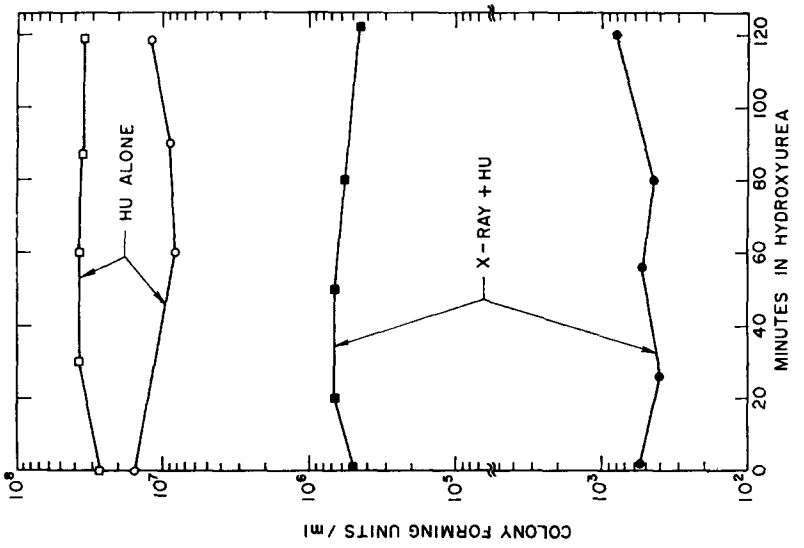

Figure 5. Post-irradiation treatment of *E. coli* K-12 *recA56* cells with 0·1 M impure HU (Nutritional Biochemicals Corp.) in SMM at 37°C. The number of viable cells is plotted as a function of time in 0·1 M HU after x-irradiation. ○, HU alone; ●, 11·7 krads irradiation then HU treatment; □, HU alone; ■, 5·1 krads irradiation then HU treatment.

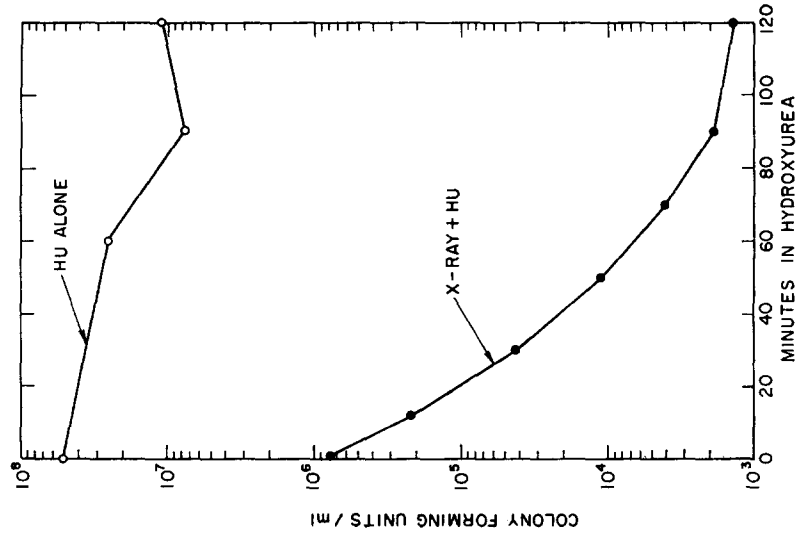

Figure 4. Post-irradiation treatment of *E. coli* K-12 *rec*$^+$ cells with 0·1 M impure HU (Nutritional Biochemicals Corp.) in SMM at 37°C. The number of viable cells is plotted as a function of time in 0·1 M HU after x-irradiation. ○, HU alone; ●, 17·6 krads irradiation then HU treatment.

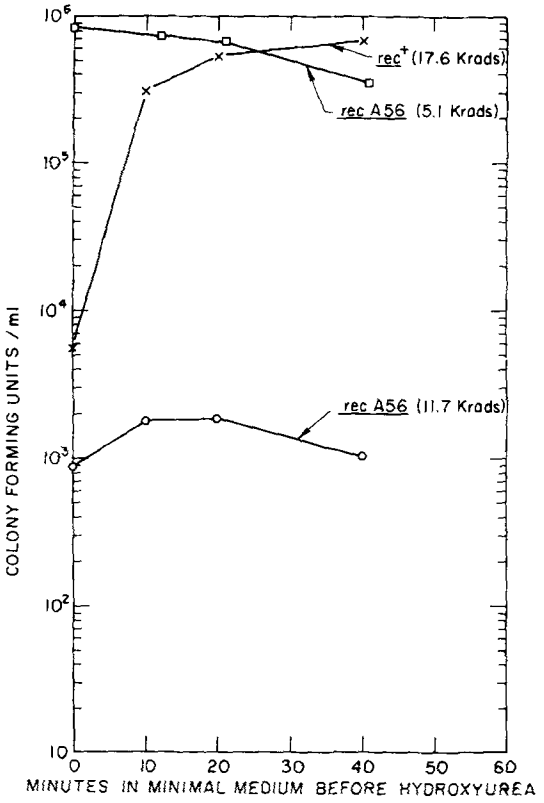

Figure 6. Effect of time of post-irradiation incubation of *E. coli* K-12 on their subsequent sensitivity to impure HU (Nutritional Biochemicals Corp.). Cells were irradiated in MM without glucose and incubated at 37°C in SMM. At the times indicated, HU was added to the cultures (final conc. 0·1 M) and allowed to act for 60 min. The number of surviving cells is plotted as a function of time of incubation before HU treatment. The *rec*+ cells received 17·6 krads and the number of surviving cells after irradiation alone was $1{\cdot}5 \times 10^6$ cells/ml. (surviving fraction (S.F.) $= 1{\cdot}4 \times 10^{-2}$). The *recA*56 cells received either 11·7 or 5·1 krads and the number of survivors after irradiation alone was $1{\cdot}8 \times 10^3$ cells/ml. (S.F. $= 1{\cdot}3 \times 10^{-4}$) or $8{\cdot}2 \times 10^5$ cells/ml. (S.F. $= 3{\cdot}7 \times 10^{-2}$), respectively. Control studies (not shown here) indicate that less than a two-fold increase in viable cells results when the irradiated (17·6 krads) *rec*+ cells are held in SMM for periods of up to 40 min prior to plating.

rec+ cells. The table demonstrates that only the chromatographically-impure HU (Nutritional Biochemicals Corporation) potentiated killing.

In a second type of experiment, the cells were allowed to repair the x-ray-induced single-strand breaks for varying periods of time before treatment with impure HU for 60 min. Figure 6 illustrates the response of the *rec*+ strain and the *recA*56 mutant. The irradiated *rec*+ cells show a dramatic loss of sensitivity to HU during incubation in SMM. By 40 min they have almost completely returned to their pre-irradiation levels of sensitivity to HU. The time course for the loss of sensitivity to HU after irradiation is similar to that for the rejoining of single-strand breaks in the *rec*+ strain (Kapp and Smith 1970 a). In contrast, no significant enhancement of survival of the *recA*56 cells appears to be produced

Irradiation (krads)	HU used		Incubation time (min)	Viability (colony-forming units/ml.)
	Manufacturer	Lot number		
None	None added	—	0	$1{\cdot}1 \times 10^7$
None	K & K Laboratory	87331F	90	$5{\cdot}8 \times 10^7$
None	Pierce Chemical Company	10018–1	90	$3{\cdot}4 \times 10^7$
None	Nutritional Biochemicals Corporation	2247	90	$1{\cdot}3 \times 10^7$
24·6	None added	—	0	$1{\cdot}7 \times 10^5$
24·6	K & K Laboratory	87331F	90	$1{\cdot}9 \times 10^5$
24·6	Pierce Chemical Company	10018–1	90	$3{\cdot}1 \times 10^5$
24·6	Nutritional Biochemicals Corporation	2247	90	$6{\cdot}8 \times 10^2$

Effect of HU from different manufacturers on viability †

† *E. coli rec*$^+$ cells (AB2497) in logarithmic growth phase were incubated at 37°C in supplemented minimal media containing 0·1 M HU. Samples were taken after 90 min, diluted and plated on SMM–agar.

by incubation in SMM before treatment with HU at either dose of irradiation, which is consistent with the observation that *recA* mutants cannot repair single-chain breaks (Morimyo *et al.* 1968, Kapp and Smith 1970 a).

Results obtained for a *uvrB*5 *rec*$^+$ mutant (not shown) were similar to those for the *rec*$^+$ strain shown in figures 4 and 6. This indicates that a mutation at *uvrB*5 has little effect on the sensitivity of x-irradiated cells to HU and is consistent with the finding that *uvr* mutations have little effect on aerobic radiosensitivity or the repair of single-strand breaks in DNA (Kapp and Smith 1970 a).

3. Discussion

Certain samples of chromatographically-*impure* HU, but not pure samples, *irreversibly* prevent the repair of x-ray-induced single-strand breaks in the DNA of *rec*$^+$ cells of *E. coli* K–12 when added after irradiation; they greatly reduce the viability of irradiated *rec*$^+$ cells, yet they produce no detectable sensitization of irradiated repair-deficient *recA* cells. If *rec*$^+$ cells are first allowed to repair their x-ray-induced single-strand breaks, they are then not sensitive to the subsequent addition of impure samples of HU.

It should be stressed that, for an agent to produce radiosensitization by the inhibition of single-strand break repair, this inhibition must be irreversible. If the inhibition is reversible, the cells can repair their breaks once they are plated in the absence of the agent and little if any effect on viability would result. This probably explains the lack of sensitization of *rec*$^+$ cells by pure samples of HU.

The employment of the *rec*$^+$ strain and the *recA*56 mutant of *E. coli* K–12, as described in this communication, provides a rapid screening technique for additional drugs that may sensitize cells to irradiation by *irreversibly* blocking repair and therefore prove useful in radiotherapy. Preliminary experiments indicate that both acriflavine (Kapp and Smith 1970 b) and quinacrine (Fuks and Smith, unpublished data) belong to this class of post-irradiation radiosensitizers. Additional experiments are now in progress to identify the impurity present in the HU responsible for the biological activity described here.

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On discute une classe de radiosensibilisateurs qui inhibent irréversiblement la réparation de ruptures d'une seule chaîne induites dans l'ADN par les rayons x. Ces agents sont actifs lorsqu'ils sont introduits après irradiation; ils augmentent l'effet létal des rayons x pour les souches *rec*$^+$ de *E. coli* K–12, mais ont peu d'effet sur la viabilité des souches irradiées *recA*, dont le pouvoir de réparation de ruptures radio-induites d'une seule chaîne est défectif. Des échantillons chromatographiquement impurs d'hydroxyurée (mais non pas des échantillons purs) ont les propriétés associées à cette classe de radiosensibilisateurs post-irradiatoires.

Es wird eine Gruppe von Strahlensensibilatoren diskutiert, die irreversibel die Reparatur von durch Röntgenstrahlen verursachten Einzelstrangbruechen in der DNS verhindern. Substanzen dieser Gruppe reagieren, falls sie nach Bestrahlung zugesetzt werden, durch Erhöhung des Strahlenschadens in *rec*$^+$-Stämmen von *E. coli* K-12. Sie haben nur geringen Einfluß auf die Überlebensfähigkeit von röntgenbestrahlte *recA*-Stämmen, die kaum Fähigkeit zur Reparatur von durch Röntgenstrahlenn verursachten Einzelstrangbrüchen besitzen. Nicht Chromatographisch reine Proben von Hydroxylharnstoff (nicht jedoch die gereinigte Substanz) zeigen die Eigenschaften dieser Gruppe von nach Bestrahlung wirksamen Sensibilatoren.

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