Influence of Hydroxyurea, Ellman's Reagent and Potassium Ferricyanide on the Radiosensitivity of *Escherichia coli*

The primary chemical lesions in DNA involved with radiation lethality of bacteria are now believed to be single-strand and double-strand breaks in DNA as well as chemical changes of the bases[1,2]. Direct chemical evidence exists for the repair of single strand and double strand breaks in bacterial DNA[3,4], although the relationship between repair of breaks and radiation survival is not yet clear.

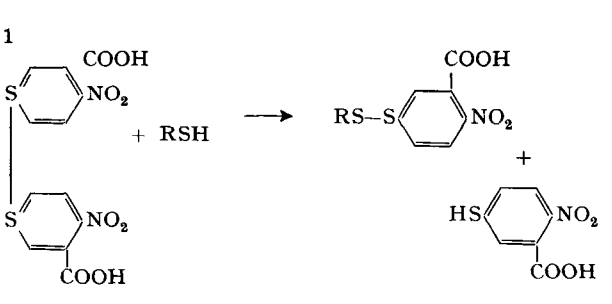
As is well known, the presence of chemical protectors or sensitizers during X-radiation can markedly influence the degree of bacterial survival as shown by changes in the slope of the irradiation dose-survival curve[5,6]. The resulting dose modifying factor (dmf), or ratio of slopes of the treated to untreated survival curves is usually of the order of a factor of 2–4 for both protection or sensitization. The best known chemical radio-protectors are amino thiols, the mechanisms of action of which have been recently discussed by BROWN[7], who suggests they bind to and stabilize DNA not covered by histones, thus reducing the DNA replication rate so that repair processes can act before the cell is called upon to divide, while the relative effectiveness and mechanisms of action of various radiosensitizers have been reviewed by BRIDGES[5]. The best known chemical radiosensitizers include iodoacetate (IAA), iodoacetamide (IA), N-ethylmaleimide (NEM), and hydroxymercuribenzoate (HMB), which all have the common feature of binding with thiol (SH) groups.

Thiol binding agents may serve as radiobiological sensitizers by blocking endogenous thiol-containing repair enzymes, thus reducing enzymatic repair[8] of radiation damage. According to this hypothesis, sensitizers should be equally effective added either before or after irradiation, but this is not usually the case, and sensitizers are most effective only when added before irradiation. Those bacterial strains possessing enzymatic repair systems should be most capable of sensitization, and this has generally been found to be true.

Chemical sensitizers may also cause the formation of a stable negative radical-ion by reaction with the radiation produced hydrated electron, allowing a greater number of lethal reactions to take place[9], or there may be formation of short-lived, toxic radiolytic products from the sensitizer molecule which can kill the bacteria[10].

Upon examining the list of thiol binding agents found to be sensitizers, it puzzled us to note that 2 compounds widely used in the quantitative analysis of protein thiol groups i.e., 5, 5′-dithiobis(2-nitrobenzoic acid) (DTNB) or ELLMAN's reagent[11], and potassium ferricyanide (PF)[12], have not yet been reported for possible radiosensitizing activity. These 2 compounds are strong oxidizing agents capable of converting the thiol groups in protein to disulfides, they in turn being reduced, as shown in equations 1 and 2 (unbalanced):

1



2 $K_3Fe(CN)_6 + 2RSH \rightarrow RS\text{–}SR + K_4Fe(CN)_6$

In order to further test the generality of radiosensitization by thiol binding agents, we have examined DTNB and PF as possible radiosensitizers, and report here our results with the radioresistant bacteria *E. coli* B/r (CSH), and with the radiosensitive bacteria *E. coli* B_{s-1} (Hill), 2 strains sensitized by NEM under anoxic conditions[13].

Since inhibitors of DNA synthesis may be expected to have an effect on cellular repair processes which also can influence radiation survival, we include here some preliminary results with hydroxyurea (HU) as a bacterial radiosensitizer. Sinclair has found that hydroxyurea sensitizes Chinese hamster cells to X-rays[14], while JACOBSON reports HU is a radioprotector of *E. coli* K12[15].

Solutions of these compounds were individually freshly prepared in saline phosphate buffer (pH 6.8) and preliminary toxicity studies with *E. coli* B/r (CSH) and B_{s-1} were carried out to assure use of a drug concentration without toxic effect on unirradiated or irradiated bacteria. From this study concentrations employed were $10^{-2}M$ with DTNB and HU, and $10^{-3}M$ with PF. Experiments with irradiated solutions of these compounds added to unirradiated bacteria were also carried out to determine whether radiolytic products toxic to the bacteria were produced the results being negative for all 3 compounds. *E. coli* B/r (CSH), having a D_0, or dose to inactivate 63% of the bacterial population (anoxic) of 23 krads, and *E. coli* B_{s-1}, with a D_0 (anoxic) of 3 krads, were grown from a loop in nutrient broth (Difco) with aeration at 37 °C to the middle or end of log phase (4 h) yielding an approximate titer of 5×10^8 cells/ml. The cells were refrigerated overnight, spun down, washed, resuspended in saline phosphate buffer (pH 6.8) and starved at 37 °C with aeration before irradiation for dose-survival studies. The appropriate volume of sensitizer solution or buffer for the controls was added to a total volume of 2 ml and the mixtures held at 0 °C for 30 min prior to irradiation in screw cap glass vials. Pre-irradiation bubbling with either oxygen or nitrogen was carried out during this holding period. The vials were then exposed at icebath temperature to 280 KVP X-rays from a dual beam Picker Vanguard Unit, operating at 280 KV and 20 mA, at a dose rate of 1.24 krads/min, with

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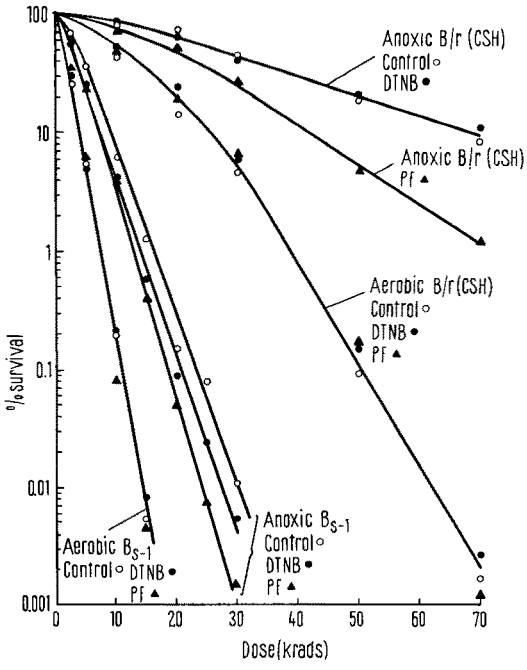

Fig. 1. Dose-survival curve for log phase *E. coli* B/r (CSH) and B_{s-1} irradiated in saline-phosphate buffer at 0 °C with 280 KV X-rays in the presence (filled symbols) and absence (open symbols) of $10^{-2}M$ 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) or $10^{-3}M$ potassium ferricyanide (PF), under anoxic and aerobic conditions.

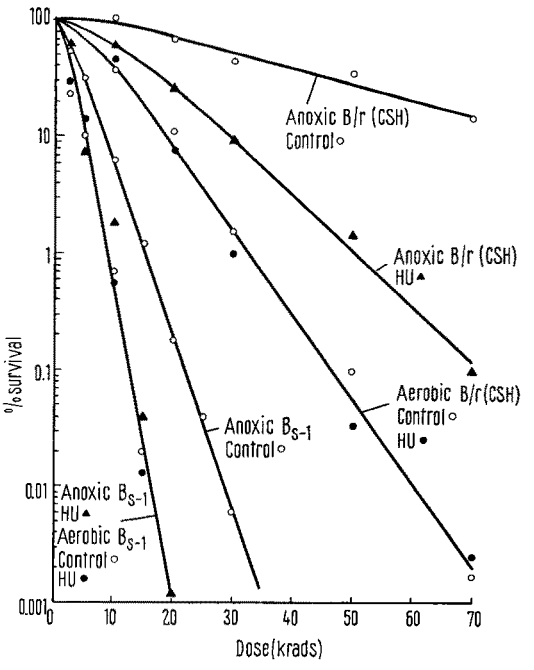

Fig. 2. Dose-survival curve for log phase *E. coli* B/r (CSH) and *E. coli* B_{s-1} irradiated in saline-phosphate buffer at 0 °C with 280 KV X-rays in the presence (filled symbols) or absence (open symbols) of $10^{-2}M$ hydroxyurea (HU) under anoxic and aerobic conditions.

continuous gas bubbling. Samples were withdrawn after graded X-ray doses, and after appropriate dilution the number of surviving bacteria was determined by plating on nutrient agar, incubating at 37 °C overnight, and counting the number of visible colonies produced.

Typical survival curves obtained for anoxic and aerobic *E. coli* B_{s-1} and B/r (CSH) suspensions in the presence or absence of $10^{-2}M$ DTNB or $10^{-3}M$ PF are shown in Figure 1. Under the conditions of this experiment it is seen that the thiol binding agent DTNB is not a radiosensitizer for either strain under either aerobic of anoxic conditions, while PF is a radiosensitizer under anoxia for B/r with a dmf of 2.4. The slight sensitization of B_{s-1} with a dmf of 1.2 is not considered significant. DTNB appeared to reduce the shoulder of the B_{s-1} anoxic survival curve, but the final slope was unchanged. Hence, of the 2 thiol binding agents investigated PF was a radiosensitizer while DTNB was not. Irradiated solutions of PF or DTNB were found to have no effect on bacterial survival of either strain when bacteria were added within 10 sec after drug irradiation. We may observe that a major difference between these 2 thiol poisons is the relatively large size of the DTNB molecule compared with PF. DTNB may not act as a radiation sensitizer despite its thiol binding capacity because of its size which may inhibit penetration to sensitive sites in the bacterium. We have no direct evidence on this point however. In the presence of oxygen, the reducing radical H· or e^-aq becomes an oxidizing species, HO_2· or O_2, and oxidizing radicals are believed more lethal to cells than reducing radicals, possibly by producing a greater amount of base damage in the bacterial chromosome. The requirement for anoxia by PF and other radiosensitizers may reflect their inability to either enhance base damage or inhibit the repair of base damage, since only base destruction is more efficient under aerobic than anoxic irradiation conditions[2] but not single strand breaks in DNA, when the effect of radiation is direct[16].

Hydroxyurea (HU) at $10^{-2}M$ (Figure 2) was also found to be an effective radiosensitizer under anoxic conditions for *E. coli* B/r (CSH) with a dmf of 3.6, and a weak radiosensitizer for *E. coli* B_{s-1} under anoxia, with a dmf of 1.7. Hydroxyurea is not considered to be a thiolbinding agent, yet it resembles thiol binding agent radiosensitizers such as PF and NEM in having a requirement for anoxia in order to sensitize, and a larger dmf with *E. coli* B/r (CSH) than with *E. coli* B_{s-1}. If inhibition of repair enzymes is involved in radiosensitization then sensitizers should be less effective with repair deficient bacteria, which is indeed observed here for PF and HU[17].

Zusammenfassung. Zwei thiolbindende Agentien, $K_3Fe(CN)_6$ und ELLMANS Reagens wie auch der die DNA-Synthese hemmende Oxyharnstoff wurden als mögliche bakterielle Sensibilisatoren für Röntgenstrahlen untersucht. $K_3Fe(CN)_6$ und Oxyharnstoff im Medium können die Bakterien für die Inaktivierung durch Röntgenstrahlen besonders sensibilisieren (stärker mit *E. coli* B/r als mit *E. coli* B_{s-1}).

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