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RADIOSENSITIZATION OF *E. COLI* BY PURINE AND PYRIMIDINE ANALOGUES INCORPORATED IN DEOXYRIBONUCLEIC ACID

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THE radiosensitivity of a given species and strain of bacteria is characteristic of that species and strain, but is subject to modification by a variety of physiological and environmental factors[1]. Among the factors which modify radiation sensitivity are oxygen[2], nitric oxide[3], and the sulphydryl-amines, such as cysteine[4], cysteamine[5] and AET[6].

A number of considerations have led investigators to postulate that the lethal effects of radiation might be mediated by damage induced in the molecular structure of the nucleic acids, in particular deoxyribonucleic acid (DNA). The discovery in recent years that certain purine and pyrimidine analogues are incorporated into bacterial DNA[7–12] makes it of interest to study the effect of the resulting alteration of molecular structure of DNA on radiosensitivity.

The work described here was initiated about a year ago[13], and is focused primarily on the X-ray sensitivity of various strains of *E. coli*, though some experiments with ultra-violet have also been performed.

Greer[14] has recently reported a striking increase of ultra-violet sensitivity in *E. coli*, strain 15*T*⁻, a thymine-deficient mutant, after incorporation of the thymine analogue, 5-bromouracil (BU). Djordjevic and Szybalski[15] have reported a similar response to ultra-violet in mammalian cells cultivated *in vitro* in the presence of 5-bromouracil deoxyriboside (BUDR), as well as the corresponding iodinated compound (IUDR). These authors also reported limited experiments with X-rays, indicating increased lethality in mammalian cells the DNA of which was labelled with these halogenated deoxyribosides.

We have used the wild-type *E. coli* strains *B*

($W3292$, radiosensitive) and B/r (radioresistant[16]); a thymine-deficient mutant of strain B ($W4516$); and a purine- and thiamin-deficient mutant of strain $K12$ ($W3687$), all obtained through the kindness of Dr. Esther Lederberg of the Department of Genetics, Stanford University.

All incubations were carried out under aerated conditions at 37° C. The mutant strains were grown from slants in a fortified medium[17] to which thymine (strain $W4516$) or purines (strain $W3687$) were added. Primary cultures of strains B and B/r were grown on mineral medium[18]. Secondary cultures employed mineral medium to which 2 per cent sulphanilamide was added to block *de novo* purine and pyrimidine synthesis. The sulphanilamide medium required supplementation with several other bases, vitamins and minerals. The purine and pyrimidine analogues under investigation were usually added at a level of 100 μgm./ml. Analogues studied to date include BU, IU, 5-fluorouracil (FU), thioguanine (TG), 2-aminopurine (AP), and 2,6-diaminopurine (DAP).

X-irradiation was carried out at 250 kV., 15 m.amp., 30 cm. distance, 0·25 mm. copper + 1·0 mm. aluminium filter, 1·10 mm. copper half-value layer, dose-rate 360–390 r./min. Cultures were collected by centrifugation, washed, suspended and diluted in phosphate buffer to concentrations of about 1×10^7 organisms/ml., and irradiated in 'Teflon' cups fitted in a 'Lucite' block, as described by Gunter and Kohn[19]. Ultra-violet irradiations were performed with an unfiltered low-pressure mercury lamp with a measured output of 16·6 ergs/sq. mm./sec. at 43 cm. distance. Samples were withdrawn at successive time-intervals from an open Petri dish buffer suspension. After irradiation, samples were appropriately diluted and plated on nutrient agar (fortified with thymine or adenine for the mutant strains) and incubated at 37° C. Colony counts at each radiation dose-level are expressed as a fraction of the viable counts of non-irradiated samples. Radiosensitivity is expressed in terms of the experimental/control slope ratios of dose – log survival curves.

DNA was isolated from aliquots of control and experimental cultures by differential chemical extraction; mixed nucleic acids by lysis with 2 per cent sodium lauryl sulphate. When necessary, RNA was removed by alkaline hydrolysis. BU incorporation into DNA was established by three different methods: cæsium chloride density gradient determination[20]; incorporation of radioactive ^{82}Br–BU (synthesized by Dr. Joseph P. Kriss[21]); and base hydrolysis and

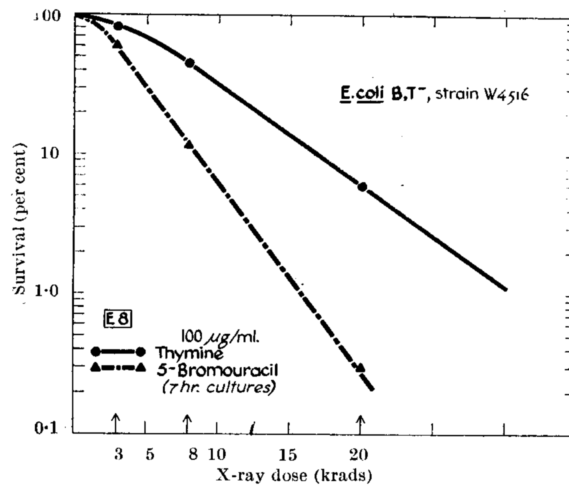

Fig. 1

chromatography. TG incorporation was determined with the analogue labelled with sulphur-35. Detection of DAP incorporation into DNA required the use of carbon-14-labelled analogue.

The incorporation of ^{82}Br–BU into DNA was linear with time until the end of the phase of exponential growth, by which time there was 35–50 per cent replacement of thymine by BU. Stationary phase cultures showed further incorporation with a plateau at about 70 per cent replacement of thymine by BU. Base hydrolysis and chromatographic analysis of DNA from stationary phase cultures indicated 68 per cent replacement. The flotation density of the isolated DNA was 1·763 (control 1·712), corresponding to 70 per cent replacement of thymine by BU. There was little or no decrease of viability of organisms that incorporated BU through the log phase; but a rapid decrease in viability with further BU incorporation during stationary phase was noted. For this reason most of the radiation experiments were carried out on organisms collected during the log phase.

Strains *W*4516 and *B*/*r* labelled with BU exhibited a striking enhancement of sensitivity to ultra-violet, the curves being essentially identical to those reported by Greer[14]. The thymineless mutant, strain *W*4516, showed a multi-hit type of X-ray dose-survival curve, with an extrapolation number which fluctuated somewhat but was usually in the range 1·5–2·0.

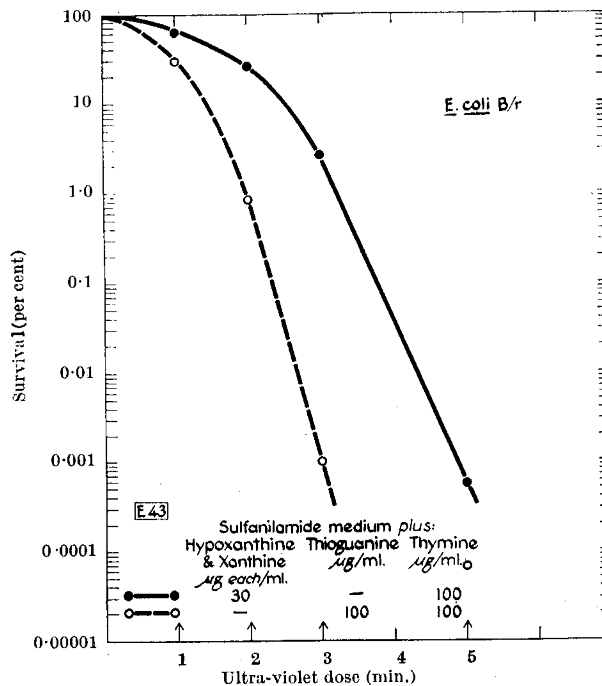

Fig. 2

When this organism was grown on BU, the shoulder of the X-ray survival curve narrowed appreciably, and in some instances vanished, while the slope of the linear portion of the curve exceeded that of the control curve by a factor of 1·8–2·2 in various experiments (Fig. 1). For the wild-type strains, the control curves of which were of the single-hit type, radiosensitization by BU yielded slope ratios of approximately 1·6–1·8 for strain *B/r* and 1·4–1·5 for strain *B* under log-phase conditions. Organisms grown on reciprocal 9 : 1 mixtures of thymine and BU exhibited responses intermediate between the extremes of 100 per cent thymine- or 100 per cent BU-grown cultures. Radiosensitization was a function of incubation time in the presence of BU, being undetectable after 2 hr. (8 per cent thymine replacement), barely detectable at 4 hr. (about 20 per cent replacement), and maximal by 8 hr. (approximately 40 per cent replacement). The possibility that some threshold-level of BU incorporation is required merits

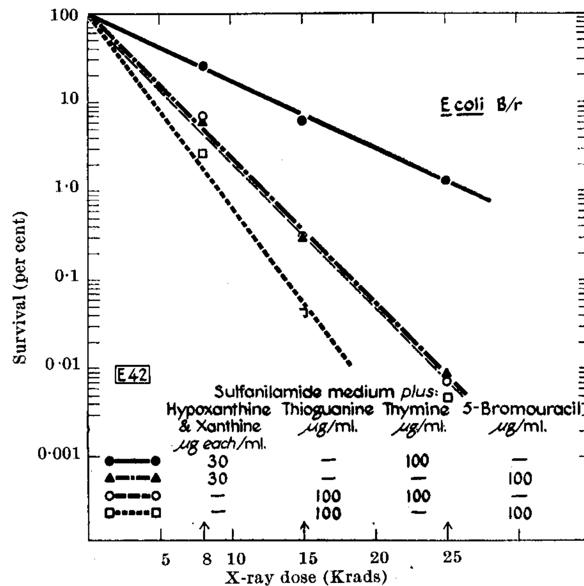

Fig. 3

further study in the time-interval between 2 and 4 hr. When the thymineless mutant strain *W*4516 was grown through log phase in BU, then diluted ten-fold and re-incubated in thymine long enough to permit one replication of DNA, the resulting organisms, containing DNA which was presumably labelled in only one strand with BU, exhibited a radiosensitivity intermediate between that of controls and fully BU-labelled cells. A somewhat greater loss of radiosensitivity has been reported in BUDR-labelled mammalian cells after one replication in the presence of thymidine[15].

In one experiment, IU-grown strain *W*4516 cells exhibited conversion to a single-hit type of curve, but the slope was essentially identical to that of the controls. In the same experiment, cells grown on BUDR showed slightly greater radiosensitization than BU-grown cells, with slope ratios of 1·80 versus 1·35 relative to thymine-grown controls, respectively. The mere presence of the analogues in the culture medium, under conditions in which incorporation into DNA could not proceed (for example, in the absence of sulphanilamide) yielded radiation dose-survival curves identical with those of controls. In limited trials to date, pre-incubation of strain *B/r*

with 5-FU has revealed no radiosensitization. This analogue, unlike IU and BU, is incorporated into RNA, but not into DNA (ref. 22).

Thioguanine is incorporated into log-phase strain *B/r* to the extent of about 0·6 per cent, calculated on the basis of replacement of DNA guanine. Although the extent of incorporation is considerably less than that of BU at corresponding time-intervals, the degree of radiosensitization to both ultra-violet and X-rays was approximately the same (Fig. 2). Consistent with this observation is the finding (to be reported in detail in a separate paper) that aqueous solutions of TG were appreciably more sensitive than any of the natural bases to spectral degradation by ultra-violet, whereas BU was only slightly more sensitive than thymine. The spectral degradation of TG by X-rays was markedly more sensitive than that of guanine and essentially identical with the responses of BU and thymine. Since purines and pyrimidines are presumably incorporated into DNA more or less independently, the effect of combined BU and TG was studied in sulphanilamide-grown strain *B/r*. In the presence of TG, BU incorporation was markedly reduced, reaching a level of only 12 per cent thymine replacement, as compared with 70 per cent in the absence of TG. None the less, organisms which had incorporated both analogues into their DNA exhibited a further increase in X-ray sensitivity (slope ratio 2·95 versus 2·2 for TG or BU alone, Fig. 3).

Although most of the radioactivity of DAP labelled with carbon-14 incorporated into DNA of purine-deficient strain *W*3687 cells was in guanine and adenine, the incorporation of unchanged DAP to the extent of about 1 per cent was demonstrable by careful chromatographic separation from guanine in the presence of carrier DAP. Replicate experiments with this strain indicated a slight increase in radiosensitivity, with slope ratios in the range 1·15–1·25. There was a departure from linearity at higher dose-levels, indicating the presence of a small proportion of cells which had failed to incorporate the analogue. In one experiment with AP in strain *W*3687, neither radiosensitization nor incorporation into DNA was demonstrable.

In summary, it appears that both ultra-violet and X-ray sensitivity of *E. coli* are enhanced when appreciable proportions of the natural bases of DNA are substituted by any of several purine and pyrimidine analogues. Analogues which fail to enter bacterial DNA (FU, AP) have to date failed to elicit this response. Based on relative extent of incorpora-

tion, TG is the most active radiosensitizing agent thus far reported, with BUDR, BU, IU, and DAP following in order of decreasing activity.

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