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Sensitization of *Escherichia coli* C to gamma-radiation by 5-bromouracil incorporation

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Escherichia coli C cells, unifilarly substituted with 5-bromouracil (BrUra) were 2·25 times as sensitive as unsubstituted cells to killing by γ-irradiation under aerobic conditions. The yield of DNA double-strand breaks in BrUra-substituted cells was increased by a factor only 1·55, suggesting that other lesions also contribute to cell-killing. Alkaline sucrose density gradient analysis of the ^{3}H-thymine labelled DNA strand showed there was less repair of γ-ray-induced single-strand breaks when BrUra was in the complementary strand. Since there are more of these unrepaired breaks than can be accounted for by BrUra-induced DNA double-strand breakage, some fraction of the lethal events in BrUra-substituted *E. coli* cells may be unrepaired DNA single-strand breaks.

1. Introduction

Sensitization to ultra-violet and ionizing irradiation occurs when 5-bromouracil is incorporated into the DNA of prokaryotic and eukaryotic cells (for reviews see Okada 1970, Hutchinson 1973).

Zimbric, Ward and Myers (1969 a, b) have shown that BrUra undergoes dissociative electron capture when irradiated, producing the highly reactive uracilyl free radical. When BrUra-substituted DNA is irradiated, the uracilyl radical can either abstract H· from an adjacent sugar, resulting in a single-strand break, or become stabilized as uracil by abstracting H· from another species (such as R-SH) in the milieu of the target. In fact, BrUra sensitization of bacteriophage T7, which is due to an increase in pyrimidine base damage Freifelder and Freifelder 1966), may be a manifestation of the conversion of BrUra to uracil.

Early work by Kaplan (1966) provided evidence that DNA double-strand breakage was increased when *E. coli*, fully substituted with BrUra, was X-irradiated, and these lesions correlated well with the increase in cellular radiosensitization. Sawada and Okada (1972) reached a similar conclusion using cultured mammalian cells. Other investigators could not establish such a correlation using either *E. coli* (Lett, Parkins, Alexander and Omerod 1964) or *Micrococcus radiodurans* (Lett, Caldwell and Little 1970) as test organisms. They favoured the hypothesis that BrUra sensitization is a result of the interference of cellular repair systems by this analogue. Although it is not clear how such an interference could occur, recent work by Grivell, Grivell and Hanawalt (1975) indicates that BrUra-substituted DNA is metabolically less stable than unsubstituted DNA, and shows a several-fold higher level of DNA turnover. These normal turnover processes may compete significantly with the repair of radiation-induced lesions, resulting in increased cell-killing. The purpose of this investigation is to define more clearly the relationship between BrUra-sensitized DNA double-strand breakage and cell inactivation.

2. Materials and methods

2.1. *Bacteria and culture conditions*

E. coli C *thy* 321 (kindly provided by Dr. R. Hewett) was used throughout this study because of its ability to tolerate BrUra substitution (Hewitt, Suit and Billen 1967). A minimal salts medium (Ganesan and Smith 1968) was supplemented with thiamine (0·5 μg/ml), thymine (10 μg/ml), and the amino acids histidine, arginine, proline, leucine, and threonine, each at 0·01 M. This growth-medium is designated SMM. Overnight cultures were diluted 1 : 100 into fresh medium supplemented with ^{3}H-methyl-thymine (New England Nuclear Corporation, 12 Ci/mmol) at 200 μCi/ml for cells used for the determination of double- and single-strand breakage. Unlabelled thymine was added to labelled cultures to a final concentration of 2 μg/ml. Cells were grown at 37°C for several generations to a density of about 2×10^{8} cells/ml, harvested by membrane filtration (0·45 μm pore-size, Millipore Corporation), washed with and resuspended in fresh growth medium containing either thymine or BrUra at 5 μg/ml, and the cell density adjusted to 10^{8}/ml. From this point, all manipulations were performed under a single 40 W incandescent yellow ' Bug-Lite ' lamp (G.E.) to prevent BrUra photolysis.

2.2. *Irradiation conditions*

Cells were collected by membrane filtration and resuspended at room temperature in DTM buffer (minimal salts medium without glucose or supplements), then chilled on ice and irradiated in a water-jacketed glass tube maintained at 3°C. Cell suspensions were aerated for 5 min before and during irradiation. Gamma-irradiation was performed with an 8000 Ci ^{137}Cs source (J. L. Shepard & Associates) at a dose-rate of 5·66 krad/min (Bonura, Youngs and Smith 1975 b).

2.3. *Survival curves*

For survival measurements, irradiated cells were diluted in 0·067 M phosphate buffer pH 7·0, and plated on SMM plates solidified with 1·6 per cent Difco Noble agar. The plates were incubated for 48–72 hours at 37°C to allow for colony formation.

2.4 *Sedimentation experiments*

Irradiated cells were either transformed to spheroplasts and lysed on neutral sucrose gradients (Bonura *et al.* 1975 a), or lysed directly on alkaline sucrose gradients (Youngs and Smith 1976). Sedimentation conditions were chosen so as to eliminate complications arising from speed dependence. Details of the molecular weight calculations have appeared elsewhere (Bonura *et al.* 1975 a, Youngs and Smith 1976).

2.5. *Equilibrium centrifugation*

In our experiments, survival and DNA single- and double-strand breakage determinations utilized the same cell preparations. Additionally, aliquots of these cell suspensions were frozen, and lysates were analysed on neutral CsCl gradients, using a procedure similar to that given by Kanner and Hanawalt (1968) to confirm that the labelled DNA molecules did contain BrUra. Frozen aliquots (0·1 ml) of cells in DTM buffer were thawed and added to a reaction mix containing 20 μl of 1 M NaCl, 0·7 ml of 0·02 M Tris-0·02 M EDTA

pH 7·6, 0·1 ml of lysozyme (1 mg/ml in 50 mM Tris pH 7·0) and 10 μl of Pronase (5 mg/ml in H_2O). After 15 min of incubation at 37°C, 10 μl of Sarkosyl NL30 was added to clarify the suspension. The cell lysate was added to a CsCl solution (7·6 g of CsCl dissolved in 5 ml of 0·02 M Tris-0·02 M EDTA pH 7·6) and spun to equilibrium in a Beckman type 65 fixed-angle rotor at 30 000 r.p.m. for 60 hours at 20°C in a Beckman L2-50 or L2-65B ultracentrifuge. The gradients were fractionated and analysed in a manner similar to that used for the sucrose gradients described above.

3. Results

3.1. *Labelling procedure*

We have found that prolonged growth of several thymine-requiring strains of *E. coli* in the presence of BrUra resulted in a significant loss of plating efficiency, and the formation of filaments (data not shown). Similar observations have been made by other investigators (Cohen and Barner 1956, Michaelson and Mandel 1962, Jones and Hewitt 1971). Additionally, the use of ^{3}H-BrUra (100 μCi/ml; 26·8 Ci/mmol) resulted in a significant amount of DNA single-strand breakage in *E. coli* C. Experiments reported by Kaplan (1966) also showed a decrease in the single-strand molecular weight of unirradiated ^{3}H-BrUra-labelled DNA compared with controls labelled with ^{3}H-thymidine. For these reasons we chose to grow the cells to a density of $\sim 2 \times 10^8$ cells/ml in medium containing ^{3}H-thymine, followed by a 2 hour incubation in medium containing either unlabelled BrUra or unlabelled thymine. During this interval, the cells grown in thymine-containing medium doubled twice, while those cells incubated in BrUra-containing medium doubled once. Thus, under these conditions, cells grown in the presence of BrUra should have one of their DNA strands substituted (unifilar substitution), while the complementary strand should contain the radioactive label (^{3}H-thymine). To ensure that this was the case with most of the cells in the population, lysates of thymine or BrUra-substituted cells were spun to equilibrium in neutral CsCl gradients. The results demonstrate that most of the native DNA containing the labelled thymine sedimented

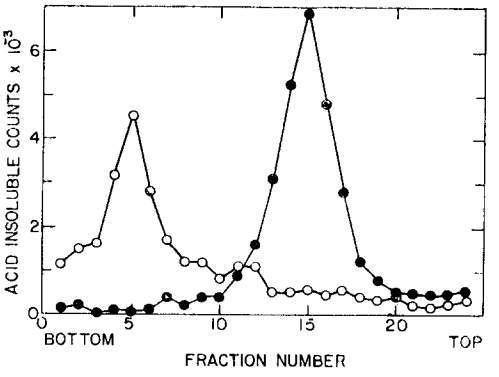

Figure 1. Equilibrium sedimentation profiles in neutral CsCl gradients of *E. coli* K-12 DNA labelled with ^{3}H-thymine for several generations and subsequently chased for 2 hours with non-radioactive BrUra (○), or thymine (●). Conditions are given in § 2. The density at the BrUra peak (fraction five) was 1·770 g/cc while the density of the thymine peak (fraction 15) was 1·710 g/cc.

to a heavy density position when BrUra was in the the growth-medium (figure 1). When the DNA from the major peaks was rebanded in alkaline caesium chloride density gradients, there was little or no material banding at densities greater than the thymine-containing strand from unsubstituted DNA, indicating that there was little or no BrUra contamination of the thymine-containing strand (data not shown).

3.2. *Survival*

Substitution of BrUra for thymine in DNA has been shown to sensitize *E. coli* to ionizing radiation by a factor of two to three (Kaplan, Zavarine and Earle 1962). Our results with wild-type cells unifilarly substituted with BrUra show a $2{\cdot}25 \pm 0{\cdot}1$-fold sensitization, based on the final slopes of the survival curves (figure 2).

3.3. *DNA strand breakage*

We measured the number of radiation-induced DNA double-strand breaks in cells that had incorporated BrUra or thymine after a labelling period in

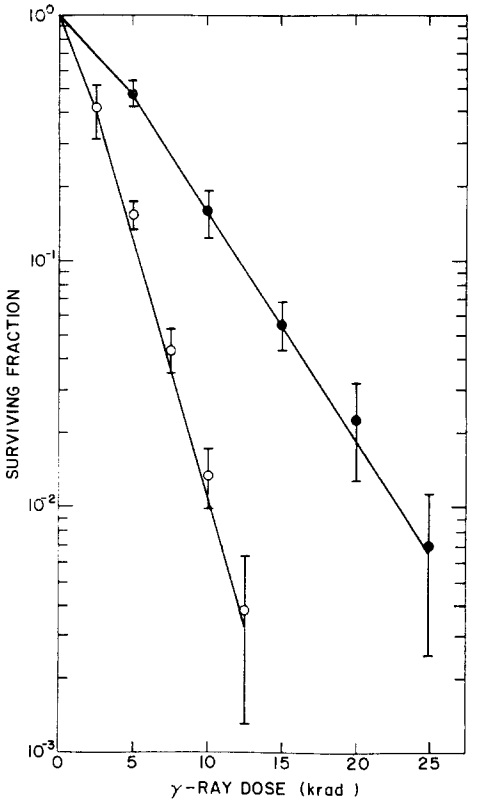

Figure 2. Gamma-ray survival curves for *E. coli* C grown in the presence of BrUra (○) or thymine (●) in log phase for 2 hours. Points represent the mean of six experiments. Lines were fitted by least-squares regression analysis excluding the control values. The D_0 values were $4{\cdot}58 \pm 0{\cdot}14$, and $2{\cdot}03 \pm 0{\cdot}06$ krad for thymine and BrUra substitution, respectively.

^{3}H-thymine. The results demonstrate that BrUra sensitizes *E. coli* DNA to double-strand breakage by a factor of $1{\cdot}55 \pm 0{\cdot}2$ (figure 3), which is significantly smaller than the 2·25-fold sensitization observed for cell-killing.

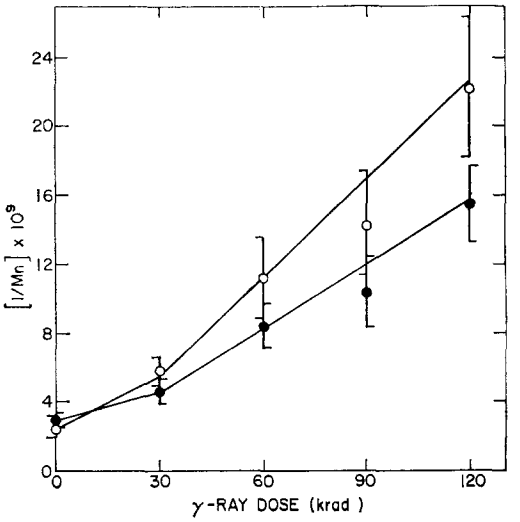

Figure 3. Production of DNA double-strand breaks in γ-irradiated *E. coli* C cells unifilarly labelled with ^{3}H-thymine and then unifilarly substituted with BrUra (○) or ^{1}H-thymine (●) as described in § 2. Points represent the mean of six experiments. Lines were fitted by least-squares regression analysis. The slopes were calculated to be $0{\cdot}11 \pm 0{\cdot}009 \times 10^{-9}$, and $0{\cdot}17 \pm 0{\cdot}016 \times 10^{-9}$ krad^{-1} for thymine and BrUra substitution, respectively.

We also investigated the production and repair of single-strand breaks in the ^{3}H-thymine-containing strand of DNA unifilarly substituted or unsubstituted with BrUra. As expected, the presence of BrUra in one strand did not affect the molecular weight of the complementary strand in unirradiated cells (figure 4 A). Irradiation with 2·5 krad, however, resulted in more breaks in the thymine-containing strand when BrUra was present in the complementary strand (figure 4 B). It is reasonable to ask if this difference could be due to the enhanced DNA double-strand breakage seen in BrUra-substituted cells (figure 3), since a fraction of the DNA single-strand breaks measured by velocity sedimentation in alkali is due to DNA double-strand breaks. The yield of DNA double-strand breaks, as calculated from figure 3, is $0{\cdot}31 \pm 0{\cdot}03$ double-strand breaks per genome per krad for cells not substituted with BrUra. This is in good agreement with the value ($0{\cdot}285 \pm 0{\cdot}02$) we reported earlier for *E. coli* K-12 W3110 (Bonura *et al.* 1975 b). The value determined for unifilarly BrUra-substituted cells is $0{\cdot}48 \pm 0{\cdot}05$ double-strand breaks per genome per krad. Assuming DNA double-strand breaks are not repaired during or immediately after irradiation, a dose of 2·5 krad should have resulted in 0·42 additional single-strand breaks per half genome, as a result of enhanced DNA double-strand breakage, above that observed in unsubstituted cells. Expressed in terms of the sedimentation profile shown in figure 4 (B), the ^{3}H-thymine-containing DNA strand from unifilarly BrUra-substituted cells would have been expected to be about 1 per cent smaller than that of unsubstituted cells if BrUra substitution only increased

the yield of double-strand breaks. The difference in molecular weights, as determined immediately after irradiation (figure 4 (B)), was actually about 19 per cent; much larger than would be expected on the basis of DNA double-strand breakage alone.

When cells irradiated with 10 krad were reincubated in SMM for 90 min after irradiation, those cells that were not substituted with BrUra showed almost complete repair of DNA single-strand breaks, whereas those cells that were unifilarly substituted with BrUra were unable to rejoin effectively single-strand breaks in their ^{3}H-thymine-containing strand (compare figures 4 (A) and (C)).

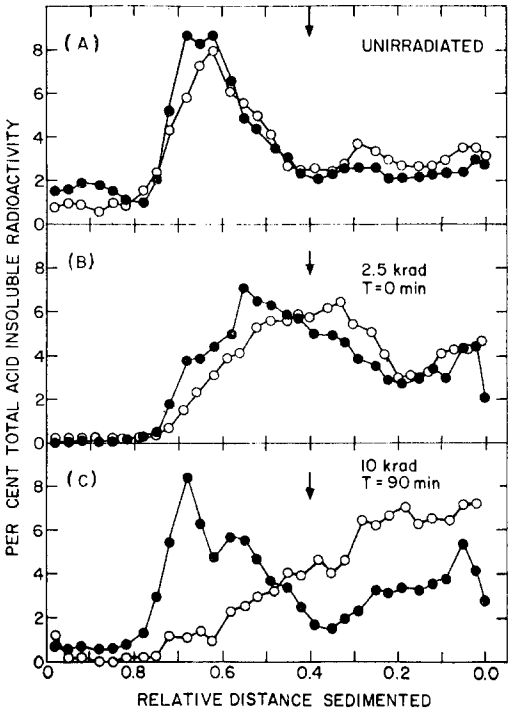

Figure 4. Alkaline sucrose gradient sedimentation profiles of ^{3}H-thymine-labelled DNA from *E. coli* C cells that were chased for 2 hours in medium containing non-radioactive BrUra (○) or thymine (●) before being irradiated. Unirradiated controls (A); 2·5 krad, lysed immediately (B); 10 krad + 90 min incubation in growth medium containing thymine (C). Note that the profiles show only the thymine-labelled strand for both BrUra-substituted and unsubstituted DNA. The position of a ^{14}C-thymine-labelled T2 phage DNA marker is shown by the arrow.

4. Discussion

Substitution of BrUra for thymine in the DNA of a variety of organisms has been shown to increase the yield of radiation-induced DNA single- and double-strand breaks (Okada 1970). Our results are in general agreement with those previously reported, but, in addition, indicate that in a cell unifilarly-substituted with BrUra the radiosensitivity of the DNA strand containing ^{3}H-thymine is influenced by the presence of BrUra in the complementary strand, i.e. DNA double-strand breakage was more frequent in DNA containing one strand

substituted with BrUra. The mechanism of DNA double-strand break formation in BrUra-substituted cells is unclear, although a number of possibilities exist. First, BrUra may sensitize DNA to double-strand breakage by single events. These might include radical-mediated events that occur in the vicinity of single-strand breaks (Bonura and Smith 1976). Second, the double-strand breaks may arise in BrUra-substituted DNA by a two-hit process, i.e. as a result of an increased yield of single-strand breaks in the substituted strand making overlapping single-strand breaks possible. Since radiation-induced base alterations (e.g., BrUra to uracil) might be more frequent in BrUra-substituted DNA (Zimbrick *et al.* 1969 b, Freifelder and Freifelder 1966), there may be an increase in the level of enzymatic excision, possibly by a uracil N-glycosidase found to be present in *E. coli* (Lindahl 1974). This activity, when coupled with an increase in radiation-induced DNA single-strand breakage, might increase the probability of two-hit, double-strand breaks.

Early work by Szybalski and Opara-Kubinska (1965) also suggested that the sensitization of transforming DNA by BrUra substitution is not restricted only to the BrUra-containing strand. A similar interpretation was proposed by Eisenberg and Pardee (1970) to explain the lethal effect of 313 nm irradiation on DNA unifilarly substituted with BrUra.

In the present experiments, radiosensitization by BrUra substitution cannot be attributable entirely to an increase in the yield of DNA double-strand breaks, since the cellular radiosensitization to killing was about 45 per cent greater than the sensitization to DNA double-strand breakage. Thus, at least in unifilarly BrUra-substituted cells, other types of damage, perhaps DNA base damage or unrejoined single-strand breaks, contribute significantly to lethality.

The number of DNA single-strand breaks present in the ^{3}H-thymine-labelled strand immediately after irradiation appears to be greater in unifilarly BrUra-substituted DNA than in unsubstituted DNA (figure 4 (B)). This difference is much larger than can be accounted for strictly on the basis of increased DNA double-strand breakage, and, therefore, may reflect a diminished ability on the part of BrUra-substituted cells to undergo the rapid repair of single-strand breaks, even in the unsubstituted strand. We cannot exclude the possibility, however, that the unsubstituted DNA strand is attacked by long-lived radicals originating from the radiolysis of the BrUra-substituted strand, nor can we conclusively say that during the period when cells were incubated in BrUra-containing medium some incorporation of the analogue into the thymine-containing strand did not occur by normal DNA turnover.

It appears that, on reincubation, there is less repair of DNA single-strand breaks in BrUra-substituted cells (figure 4 (C)). Recently, Grivell *et al.* (1975) have shown that BrUra-substituted DNA is metabolically less stable than unsubstituted DNA in *E. coli*. This instability is manifested by an increased turnover of DNA containing BrUra, presumably by a repair process (Grivell *et al.* 1975). This enhanced turnover might interfere with the effective rejoining of DNA single-strand breaks in unifilarly BrUra-substituted cells.

In conclusion, our data suggest that BrUra substitution for thymine on one DNA strand exerts an effect on the complementary strand, since the repairability of the single-strand breaks in the non-BrUra-containing strand is diminished when BrUra is incorporated into the complementary strand. The mechanism of BrUra sensitization in unifilarly substituted cells is the result

both of enhanced DNA double-strand breakage, and an enhanced yield and/or a diminished ability to repair DNA single-strand breaks and/or base damage.

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Des cellules *Escherichia coli* C, dont une chaîne d'ADN contient du 5-bromouracil (BrUra) sont 2,25 fois plus sensibles que des cellules normales aux radiations ionisantes dans des conditions aérobiques. Le nombre de cassures doubles des chaînes d'ADN n'augmente qu'avec un facteur 1,55, ce qui suggère que d'autres lésions interviennent dans l'effet léthal de l'irradiation. Une analyse de la vitesse de sédimentation de l'ADN dans des gradients alcalins de sucrose montre qu'il y a moins de réparation des cassures monocaténiques lorsqu'il y a BrUra dans la chaîne complémentaire d'ADN. Cette augmentation du nombre de cassures monocaténiques est trop grande pour être seulement due aux cassures doubles. Il semble donc que l'effet sensibilisateur du BrUra soit dû à une inhibition des processus de réparation des cassures monocaténiques.

Escherichia coli C Zellen, bei denen 5-Bromouracil (BrUra) im Einzelstrang des DNS substituiert wurde, waren für den Zelltod durch γ-Strahlung unter aeroben Bedingungen 2,25 mal empfindlicher als unsubstituierte Zellen. Der Ertrag doppelsträngiger DNS Brüche nahm in BrUra-substituierten Zellen nur mit dem Faktor 1,55 zu. Es scheint demnach, dass andere Schäden auch zum Zelltod beitragen.

Bei dem mit ^{3}H-Thymin markierten DNS Strang konnte mit Hilfe eines alkalischen Sukrose Dichtegradienten aufgezeigt werden, dass die durch γ-Strahlung entstandenen Einzelstrangbrüche in geringerem Ausmass repariert wurden, wenn der, komplementäre Strang BrUra enthielt.

Ein Teil des letalen Effekts der mit BrUra substituierten *E. coli* Zellen könnte auf diese nicht reparierte Einzelstrangbrüche zurückgeführt werden da mehr unreparierte Brüche festgestellt wurden als man für BrUra induzierte Doppelstrangbrüche errechnen konnte.

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