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Effect of Halogenated Pyrimidines on Radiosensitivity of *E. coli*[1]

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INTRODUCTION

The possibility of selective modification of cellular radiosensitivity by pretreatment with various chemical agents has received increasing attention in recent years (*1, 2*). Among those agents which have been shown to modify radiosensitivity are oxygen and nitric oxide, which have a radiosensitizing effect (*3, 4*), and the sulfhydryl amines, such as cysteine, cysteamine, and AET,[2] which exert a protective action (*5*).

A more specific approach to the problem of chemical modification of radiosensitivity has now begun to emerge, based on the postulate that the lethal effects of radiation may be mediated by damage induced in the molecular structure of cellular nucleic acids. This approach has received considerable impetus from the availability of a variety of pyrimidine analogs which have been shown to be incorporated into cellular DNA (*6–10*). A fraudulent nucleic acid is thus formed, the molecular structure of which might be altered in such a way as to enhance radiosensitivity.

Support for this possibility first emerged from the work of Greer (11), who noted a striking increase in sensitivity to ultraviolet irradiation of *E. coli* strain 15T$^-$, a thymine-deficient mutant, after it had been grown on the thymine analog BU. Djordjevic and Szybalski (*12*) noted a similar response to ultraviolet and X-irradiation in mammalian cells cultivated *in vitro* in the presence of BUDR and IUDR. In studies begun about two years ago, we have explored the effect of various purine and pyrimidine analogs on radiosensitivity of *E. coli*. A bacterial system was selected because it was felt that the enhanced radiosensitivity would, in all

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[2] Abbreviations used: AET = aminoisoethylthiouronium-Br-HBr; DNA = deoxyribonucleic acid; RNA = ribonucleic acid; BU = 5-bromouracil; FU = 5-fluorouracil; BUDR(IUDR) = BU(IU) deoxyriboside.

TABLE I

FINAL COMPOSITION OF SULFANILAMIDE MEDIUM[a]

	%		μg/ml
K_2HPO_4	0.7	Hypoxanthine	30
KH_2PO_4	0.2	Xanthine	30
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.042	Thymine	100
$MgSO_4 \cdot 7H_2O$	0.01	DL-Methionine	30
$(NH_4)_2SO_4$	0.1	DL-Serine	30
$CaCl_2 \cdot 2H_2O$	0.0005	DL-Valine	30
$FeSO_4 \cdot 7H_2O$	0.000025	L-Histidine	30
Sulfanilamide (Eastman)	0.2	Pyridoxine	1
Glucose	0.4	Calcium pentothenate	1
		Thiamine	1

[a] Modified from Cohen and Barner (*17*).

likelihood, be the composite result of the interaction of a number of parameters which could be more quickly sorted out in bacteria than in mammalian cells. Although preliminary reports of this work have appeared elsewhere (*13–15*), it seemed desirable to present the data in more complete form. This communication is concerned with halogenated pyrimidine analogs; a subsequent communication will deal with purine analogs.

MATERIALS AND METHODS

Bacterial Strains

The bacterial strains include the radiosensitive *E. coli* strain B (W3292), the radioresistant strain B/r (*16*), and a thymine-deficient mutant of strain B (W4516), all of which were kindly provided by Dr. Esther Lederberg of the Department of Genetics, Stanford University.

Culture Media

The thymine-deficient mutant was grown from slants in a fortified medium (*9*) to which thymine was added at 100 μg/ml. Primary cultures of strains B and B/r were grown from nutrient agar slants in mineral medium,[3] usually for 8 hours, and then diluted 1:50 into mineral medium supplemented with sulfanilamide (to block thymidylate synthesis, *17*), vitamins, and a purine and pyrimidine source. The final composition of the medium is indicated in Table I. All incubations were carried out under aerated conditions at 37°C. A sample growth curve for strain B/r is presented in Fig. 1.

[3] Modified from Davis mineral medium as described by J. Lederberg, *Methods in Med. Research* **3**, 5 (1950). Final composition: K_2HPO_4, 0.7%; KH_2PO_4, 0.2%; $Na_3C_6H_5O_7 \cdot 2H_2O$, 0.042%; $MgSO_4 \cdot 7H_2O$, 0.01%; $(NH_4)_2SO_4$, 0.1%; $CaCl_2 \cdot 2H_2O$, 0.0005%; $FeSO_4 \cdot 7H_2O$, 0.000025%; glucose, 0.1%.

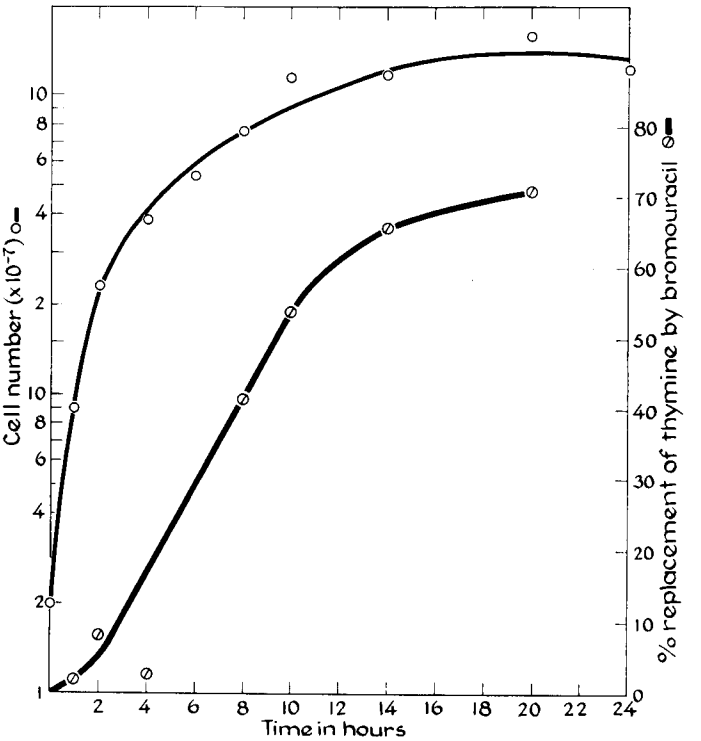

FIG. 1. Growth curve for *E. coli* B/r in sulfanilamide medium containing bromouracil and the per cent replacement of thymine by bromouracil with time. See text for further details.

Pyrimidine Analogs

BU, IU, BUDR, and FU were obtained from California Corporation for Biochemical Research, Los Angeles. They were usually added to culture media at a level of 100 μg/ml. In some experiments Br^{82}-labeled BU or BUDR, synthesized by Dr. Joseph P. Kriss of the Isotope Laboratory, Stanford University School of Medicine, was used (*18*).

Irradiation

X-irradiation was carried out at 250 kv, 15 ma, 30 cm distance, 0.25-mm Cu+ 1.0-mm Al filter, 1.10-mm Cu HVL, dose date 360 to 390 r/min. Cultures were harvested by centrifugation (10,300 $\times$ *g* for 12 minutes), washed, suspended, and diluted in 0.05 *M* K_2HPO_4–0.05 *M* KH_2PO_4 to concentrations of about 1×10^7 organisms per milliliter, and irradiated in Teflon cups fitted in a Lucite block, as described by Gunter and Kohn (*19*). Ultraviolet irradiations utilized an unfiltered low-pressure mercury lamp, with a measured output of 16.6 ergs/mm^2/sec at 43 cm

distance. Samples were withdrawn at successive time intervals after the start of exposure from a buffer suspension in an open petri dish. After irradiation, samples were appropriately diluted and plated on nutrient agar (fortified with 200 μg of thymine per milliliter for the mutant strain) and incubated at 37°C. Colony counts at each radiation dose level are expressed as a fraction of the viable counts of nonirradiated samples. Relative X-ray sensitivity of the experimental versus control cultures is expressed in terms of the ratios of the slopes of the dose-log survival curves.

Nucleic Acid Isolation and Analysis

For density gradient studies the mixed nucleic acids were isolated from aliquots of control and experimental cultures by the method of Smith and Kaplan (*20*), except that the initial heat step was replaced by a 2-hour treatment of the cells with 2% sodium lauryl sulfate at room temperature. For subsequent base analysis the RNA was removed by treatment with 1 *N* NaOH at room temperature for 24 hours and the subsequent recovery and washing of the DNA with acid.

If the DNA was required only for base or radioactivity analysis, the cells were extracted three to five times with cold 0.2 *N* perchloric acid and twice with ethanol–ether (3:1) at 60°. The RNA was hydrolyzed as above, and the DNA and protein were precipitated in 5% trichloroacetic acid and washed several times with cold 0.2 *N* perchloric acid. The DNA was extracted from the protein with 5% trichloroacetic acid for 15 minutes at 90°; the supernatant was taken to dryness under vacuum and hydrolyzed in 88% formic acid (*21*). After drying under vacuum, the residue was taken up in 1 *N* HCl, spotted on Whatman No. 1 paper, and chromatographed first in methanol–HCl (*22*) and then in *n*-butanol–1 *N* NH_4OH (6:1, v/v).

The incorporation of BU into DNA was established by paper chromatography by the above techniques. Its presence and amount could also be determined by buoyant density studies in cesium chloride (*23*). Given the density and the base composition of the control DNA, one can calculate the per cent replacement of thymine by bromouracil in the experimental sample from its buoyant density. These data agreed with those obtained by the chromatographic method.

A third technique for the measurement of the replacement of thymine by BU involved the use of radioactive Br^{82}-bromouracil. The cells were washed as described above. After the RNA was removed and the reprecipitated DNA and protein were thoroughly washed, the DNA was hydrolyzed in 0.5 *N* perchloric acid for 30 minutes at 80°. The amount of DNA in the supernatant was estimated by its absorption at 260 mμ. With the base composition of the DNA sample known, the amount of thymine per optical density unit of the hydrolyzed DNA can be calculated. The amount of BU present is obtained from the radioactivity measurements. The per cent replacement of thymine is thus easily calculated. One would

expect an inherent error in this method of about 8% due to the inequality of the extinction coefficients of BU and thymine. Nevertheless, in one experiment this type of calculation indicated 71% replacement, and the chromatographic analysis of a control culture grown in nonradioactive medium gave 68% replacement.

RESULTS

Rate of Incorporation into DNA

The rate of incorporation of BU into DNA was followed with the Br^{82}-labeled analog in strain B/r on sulfanilamide medium (Fig. 1). Incorporation was linear with time from 2 to 10 hours, at the end of which time there was about 50 to 55% replacement of thymine by BU. In the subsequent stationary phase, there was further incorporation to a plateau at about 70% replacement of thymine by BU. Base hydrolysis and chromatographic analysis of DNA from stationary phase cultures, grown on cold BU medium, indicated 68% replacement. The buoyant density of the isolated DNA was 1.763, as compared with a normal value of 1.712, corresponding to 70% replacement of thymine by BU.

Effect of the Analogs on Viability

Incorporation of BU had little effect on the viability of *E. coli* through log phase. The viable counts of unirradiated cultures containing up to 40% BU in lieu of thymine were usually from 80 to 100% of control values. However, there was a very rapid decrease in viability down to 10% or less with further BU incorporation during the stationary phase of cultivation. For this reason most irradiation experiments were carried out on organisms harvested during log phase.

Ultraviolet Irradiation

Organisms grown through log phase on BU exhibited a striking alteration of the dose-survival curve. Figure 2 indicates the nature of this response, which is essentially identical to that reported by Greer (*11*). The principal effect is a reduction in width of the "shoulder" of the curve, with only slight increase in slope of the exponential part of the curve. There was a departure from linearity at higher dose values, apparently due to the presence of a very small proportion of resistant cells.

X-Irradiation

Although the dose-log survival curves for strains B and B/r after X-irradiation are of the single-hit type, control cultures of the thymineless mutant strain W4516 showed a "multihit" type of dose-survival curve, with an extrapolation number which was usually in the range of 1.5 to 2.0, though subject to some fluctuation from one experiment to the next.

When this mutant was grown on BU through log phase, the shoulder of the X-ray survival curve diminished greatly in width, and in some experiments there

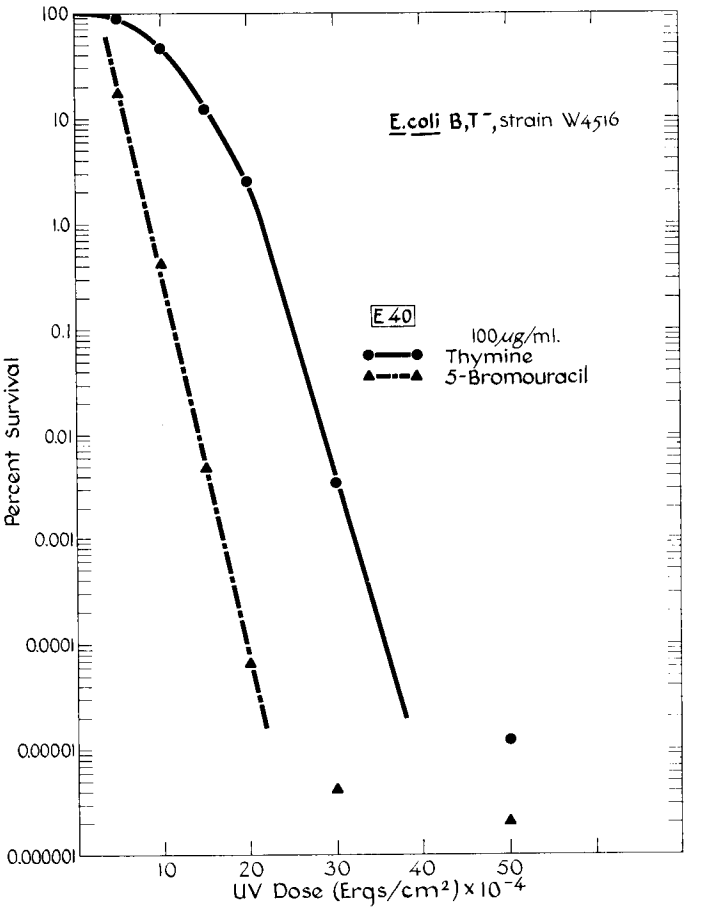

FIG. 2. The effect of ultraviolet irradiation on the survival curves of *E. coli* B,T⁻ grown on thymine or bromouracil.

was actually a complete disappearance of the shoulder. The slope of the exponential portion of the curve increased by a factor of 1.8 to 2.2 over that of controls grown on thymine (Fig. 3). In one experiment, in which the organisms were grown on BU into stationary phase and the viable count of the unirradiated analog-grown organisms was only about 10% of control levels, an X-ray survival-curve slope ratio of 2.6 was obtained, suggesting that radiosensitization was somewhat greater under these conditions.

The radioresistant strain B/r, grown on BU in the absence of sulfanilamide, failed to incorporate the analog. Under these conditions there was no significant

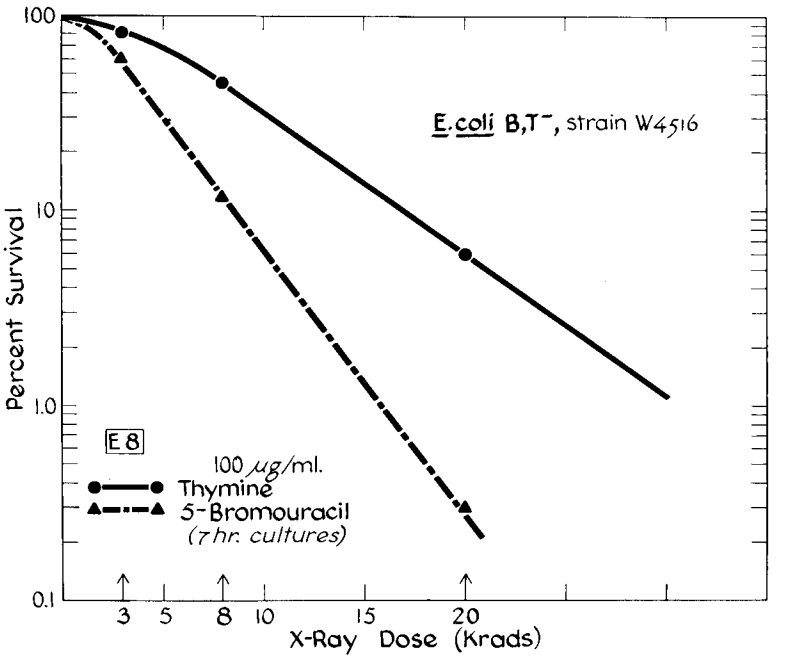

FIG. 3. The effect of X-irradiation on the survival curves of *E. coli* B,T⁻ grown on thymine or bromouracil.

radiosensitization. When sulfanilamide was added to the culture medium to block *de novo* thymidylate synthesis, added BU was incorporated into DNA, and X-ray sensitivity was concomitantly enhanced, with slope ratios of approximately 1.6 to 1.8 (Fig. 4). A similar, but lesser, response occurred with radiosensitive strain B organisms. There was no sensitization when BU was added to sulfanilamide-free culture medium; when sulfanilamide was present in addition to BU, slope ratios of 1.4 to 1.5 were obtained for strain B under log-phase growth conditions. In one early experiment BUDR added to the culture medium of strain B, in the absence of sulfanilamide, also yielded no detectable radiosensitization.

Relative Activities of the Halogenated Pyrimidines as Radiosensitizers

In one experiment, the thymineless mutant exhibited X-ray survival-curve slope ratios of 1.05 for IU, of 1.35 for BU, and of 1.80 for BUDR. Under these conditions, therefore, BU appears to be a more effective X-ray sensitizer than IU, and the deoxyriboside appears to be more effective than the free base. Erikson and Szybalski (*24*) found IUDR to be the most effective X-ray sensitizer for mammalian cells.

In other experiments, *E. coli* were grown on 10.9 μg/ml of FU, which, unlike BU and IU, is incorporated into RNA but not into DNA (*25*). There was no radiosensitization by this analog under these conditions.

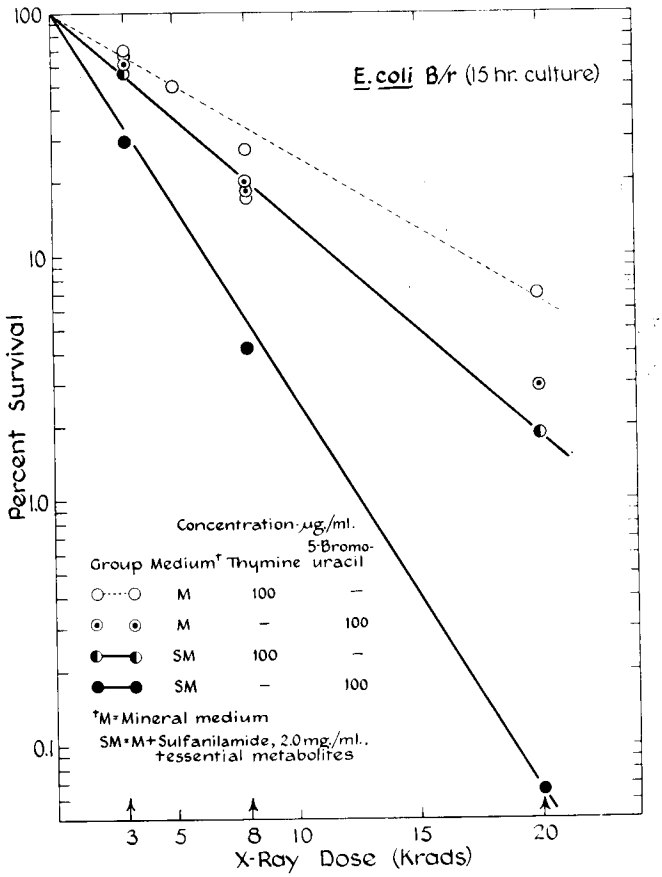

FIG. 4. The effect of X-irradiation on the survival curves of *E. coli* B/r grown in the presence of thymine or bromouracil with and without added sulfanilamide.

Radiosensitization as a Function of Extent of Analog Incorporation

The thymine-deficient mutant W4516 grown on reciprocal 9:1 mixtures of thymine and BU exhibited X-ray responses intermediate between those for 100% thymine-grown or 100% BU-grown cultures (Fig. 5). When strain B/r was grown on BU in sulfanilamide medium and aliquots were removed at serial intervals for testing of X-ray sensitivity, there was no detectable radiosensitization at 2 hours (5% thymine replacement); minimal radiosensitization at 4 hours (about 17% replacement), which was apparent at low, but not at higher, doses, suggesting that BU incorporation was not homogeneous in the bacterial population; and maximal radiosensitization by the end of log-phase growth at 8 hours (approximately 41% replacement). These time intervals correspond to 3.4, 4.2, and 5.2 doublings of the bacterial population at 2, 4, and 8 hours, respectively (Fig. 6).

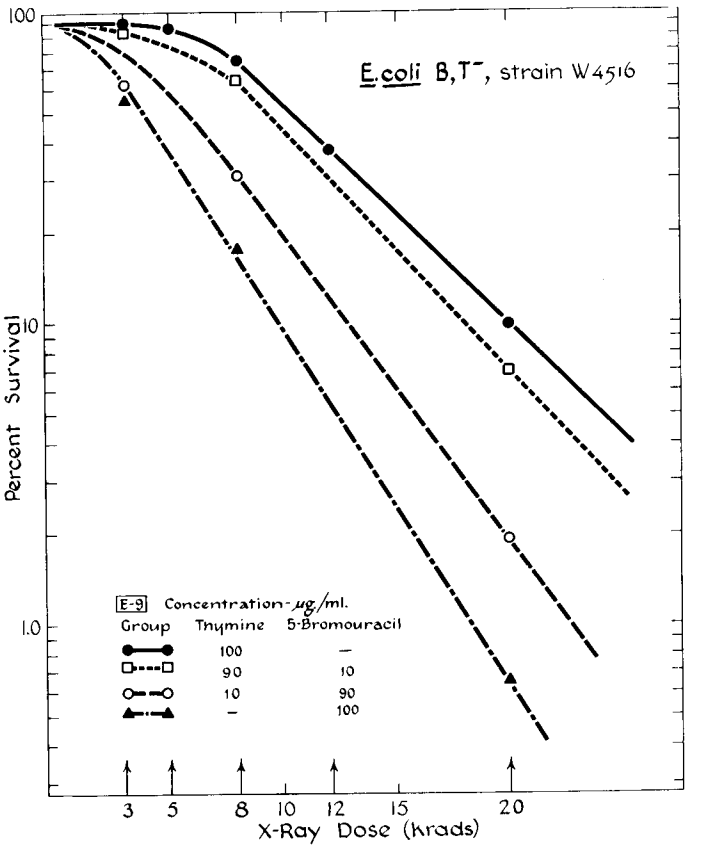

FIG. 5. The effect of X-irradiation on the survival curves of *E. coli* B,T⁻ grown on reciprocal mixtures of thymine and bromouracil.

Influence of "Bifilar" versus "Unifilar" DNA Labeling with Analog

Djordjevic and Szybalski (*12*) reported that labeling of both strands of the double helical DNA molecule with BUDR (which they termed "bifilar" labeling) is essential for enhancement of ultraviolet sensitivity of mammalian cells. Cells first grown on BUDR and then permitted to replicate their DNA once more in the presence of thymidine could be shown to contain DNA with a cesium chloride density gradient equilibrium intermediate between that of fully BU-labeled DNA and that of normal unlabeled DNA, apparently confirming the semiconservative DNA replication hypothesis (*26*). This result has been independently reported with BUDR-grown HeLa cells by Simon (*27*).

Nonsynchronous cultures of mammalian cells containing "unifilar"-labeled DNA

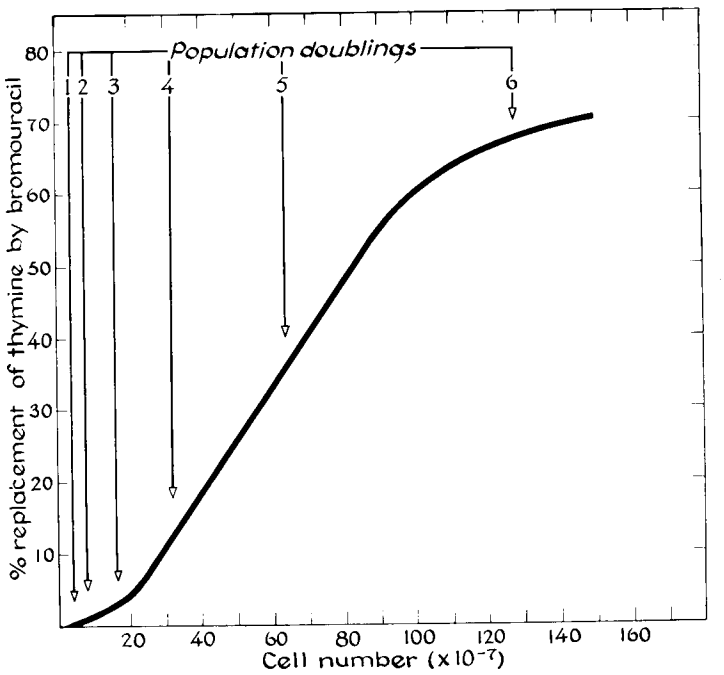

FIG. 6. Per cent replacement of thymine by bromouracil versus number of population doublings for *E. coli* B/r.

(as indicated by CsCl buoyant density) exhibited two-phase UV dose-log survival curves with a steep initial slope down to less than 1% survival, beyond which the slope diminished to that of BUDR-free cells (*12*). No data on X-ray sensitivity of cells containing "unifilar"-labeled DNA were presented.

In our corresponding experiments with *E. coli*, the "unifilar"-labeled cells exhibited responses to both X-ray and ultraviolet exposure which were intermediate betwen those of controls and "bifilar"-labeled cells (Figs. 7 and 8). Since the slopes of the UV curves are not greatly different from those of the controls, even in the case of "bifilar"-labeled organisms, this criterion is not very sensitive in the case of the bacterial experiments. The X-ray curves, however, clearly indicate that about 50% of the radiosensitization persists when only one strand of DNA is labeled. Density gradient centrifugation studies indicated buoyant equilibrium densities of 1.765 for DNA from "bifilar"-labeled cells, of 1.735 (68%) and 1.705 (32%) for DNA from "unifilar"-labeled cells, and of 1.705 for control cells. The admixture of a small proportion of light DNA with the "unifilar"-labeled DNA is attributable to the fact that the cultures were not synchronous; thus, some of the originally BU-labeled bacterial cells could have replicated their DNA twice after

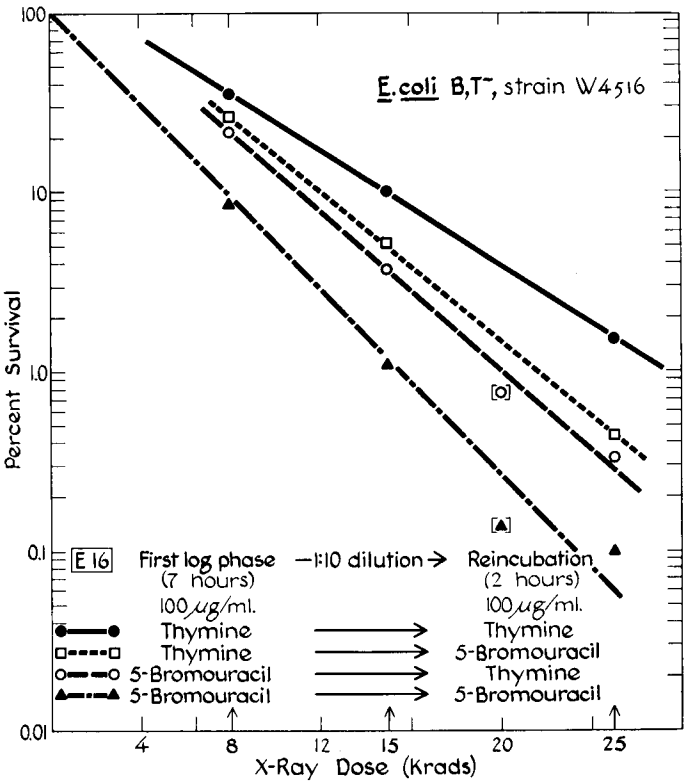

FIG. 7. Effect of X-irradiation on the survival curves of *E. coli* B,T⁻ grown on thymine or bromouracil and subcultured in reverse media for 2 hours.

transfer to thymine medium, despite the fact that the cells were harvested and irradiated after slightly less than one doubling of cell number.

DISCUSSION

The interaction of two agents presents some interesting semantic problems, which have recently been discussed by Scott (*1*). The effect observed in these experiments is clearly not synergism, since the halogenated pyrimidines were employed under conditions in which they had little or no effect by themselves on viability. The most appropriate term would appear to be sensitization or potentiation, since the increase in slope of the X-ray dose-log survival curve reflects an intrinsically enhanced response to ionizing radiation. That slope ratios greater than 2.0 can be obtained under optimal conditions indicates that the magnitude of the effect is almost as great as that due to oxygen. There is as yet no evidence to establish whether these two effects are in any way related.

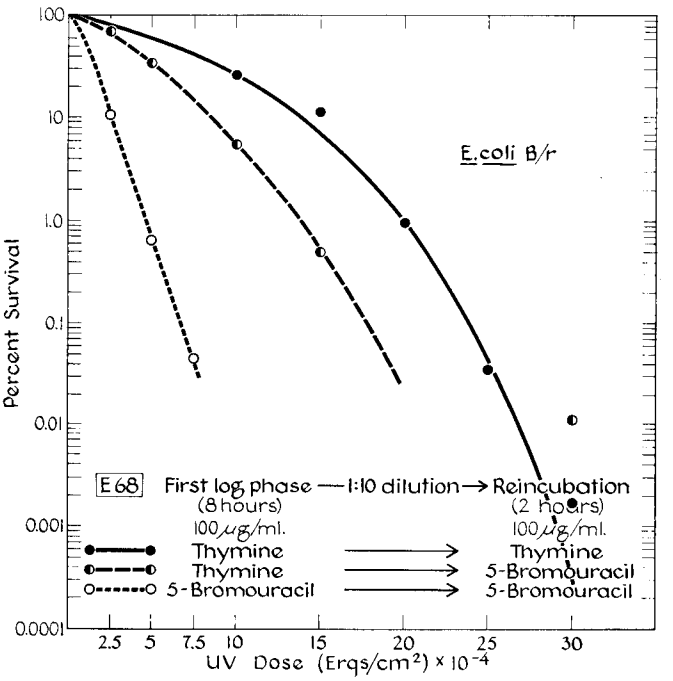

FIG. 8. Effect of ultraviolet irradiation on the survival curves of *E. coli* B/r grown on thymine or bromouracil and subcultured in reverse media for 2 hours.

It seems clear that the radiosensitization conferred by these halogenated pyrimidines is dependent on their incorporation into cellular DNA. This conclusion is supported by several lines of evidence. In the present experiments, sulfanilamide was added to mineral medium to block *de novo* thymidylate synthesis by strains B and B/r. The halogenated analogs were apparently unable to compete with the *de novo* pathway and were thus not incorporated to any significant extent into the DNA of these strains in the absence of sulfanilamide. Under these conditions, they exhibited no appreciable radiosensitization. Moreover, incorporation of BUDR was progressive with time both in our experiments and in those of Djordjevic and Szybalski (*12*), and in both the degree of radiosensitization also increased progressively with time to a plateau at about 40 to 50% substitution of BU for thymine. Other analogs which are not incorporated into DNA lack radiosensitizing activity. This is evident in our experiments with FU, as well as in other studies with purine analogs, to be published separately. Finally, radiosensitization of transforming DNA from BUDR-grown *B. subtilis* has now been reported after UV irradiation either *in vitro or* in *vivo* (*28*).

There is little information concerning the mechanism whereby these DNA-in-

corporated analogs exert their effect at the molecular level. Kit and Hsu (*29*) find that DNA-containing BUDR has a slightly higher melting temperature (T_m) (*30*) than the control. Presumably the effect of the halogen is either in strengthening the hydogen bonding with adenine or in changing the interaction of DNA with cations, since the T_m is a function of cation concentration. Recent work by Stahl (*31*) suggests that BU incorporation interferes with photoreactivation of UV-damaged bacteriophage, but the molecular basis for this effect is also unknown. It seems likely, however, that the radiosensitizing and photoreactivation-inhibiting effects will be due to closely related, and perhaps identical, mechanisms.

The potential usefulness of these analogs in clinical radiotherapy has very properly been commented on by Djordjevic and Szybalski (*12*), who point out that differential radiosensitization of neoplasms might be achieved by coupling the selective affinity of such agents for dividing (DNA-synthesizing) cells with localized irradiation, which would thus confine the enhanced effect to the treatment field and spare normal rapidly dividing tissues such as marrow and intestine. If "bifilar" labeling of DNA were really essential, however, as these authors have claimed, then the clinical utility of these agents could be dismissed *a priori*, since each tumor would have to be permitted to double in size, and then to replicate its DNA once more in preparation for a second doubling, before the radiosensitivity of any of its cells could be significantly enhanced. Fortunately, the validity of the need for "bifilar" labeling is open to serious question. The evidence is confined to experiments with ultraviolet irradiation in which distinctly two-phase dose-log response curves were obtained with "unifilar"-labeled cells. Djordjevic and Szybalski appear to base their conclusion on the fact that the distal slopes of their curves for "unifilar"-labeled cells parallel those for unlabeled cells. However, the break in slope of their two-phase curves usually occurred at less than 1% survival. The distal slope might thus be attributable, in a nonsynchronous culture, to the response of a small proportion of initially labeled cells which had replicated DNA twice, rather than only once, in BUDR-free medium and thus contained a preponderance of unlabeled DNA. This interpretation is supported in part by one of their CsCl gradient curves (curve 4, their Fig. 4*a*) for DNA extracted from such cultures, which reveals a distinct second peak at the buoyant density (1.703) of normal "light" DNA. Although no normal peak is seen in curves 2 and 3 (their Fig. 4*a*), this might be due to inability of the method to detect very small amounts of such DNA, since the corresponding UV dose-log survival curves indicate a break in slope at only 0.1 to 0.5% survival. It would thus seem reasonable to associate the presence of "unifilar"-labeled DNA in their curves 2, 3, and 4 with the steep components of the corresponding UV survival curves, which account for 99% or more of cell death. Since these steep components have a slope which parallels that of "bifilar"-labeled DNA, essentially maximal UV radiosensitization may well be conferred by BU incorporation into a single strand of DNA.

This revised interpretation of the UV data of Djordjevic and Szybalski is supported by our X-ray and UV data for "bifilar" and "unifilar" BU-labeled *E. coli*. In each case, the curves of the "unifilar"-labeled cells were intermediate between those for "bifilar" and unlabeled cells. In bacteria, therefore, at least half-maximal radiosensitization can be obtained with only one strand of DNA labeled with the halogenated analog. It would be highly desirable to eliminate any remaining uncertainties on this point by repeating both the bacterial- and mammalian-cell experiments with synchronized cultures.

Much more threatening to the prospect of radiotherapeutic exploitation of these agents are our data on the quantitative relationships between degree of incorporation, numbers of cell doublings, and degree of X-ray sensitization (Figs. 1 and 6). If corresponding studies with mammalian cells were to yield comparable results, extrapolation to clinical situations would require that tumors be permitted to double in size between three and four times in the sustained presence of the analog before appreciable enhancement of radiosensitivity could be expected. Fortunately, there is another approach which might circumvent this problem. Most radiotherapy is given in multiple fractionated doses over a period of weeks. *Alternate* use of the analogs and irradiation doses, if spaced at optimal intervals, could label and then selectively destroy a small proportion of the tumor cells at each cycle, leaving the more radioresistant cells behind to be labeled in a subsequent cycle. If such an approach enabled complete sterilization of the tumor with as little as a 10% reduction in total dose, it would represent a very real advance, since the margin of safety in many radiotherapeutic situations is small.

SUMMARY

The incorporation of halogenated thymine and thymidine analogs into *E. coli* DNA during log-phase growth, though relatively nontoxic *per se*, was associated with a significant increase in sensitivity of the bacterial cells to X-ray and UV irradiation. Although incorporation was linear with time from 2 to 10 hours of incubation, significant radiosensitization was not observed until 4 hours (17% replacement of thymine by 5-bromouracil) and was maximal at the end of log-phase growth (40 to 50% replacement), despite a further increment of incorporation during stationary phase. In contrast to other reports in which mammalian cell cultures were employed, labeling of only one strand of the DNA double helix was sufficient to confer at least half-maximal radiosensitization. The implications of these data for clinical radiotherapy are discussed.

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