BREAKAGE OF PARENTAL DNA STRANDS IN *HAEMOPHILUS INFLUENZAE* BY 313 nm RADIATION AFTER REPLICATION IN THE PRESENCE OF 5-BROMODEOXYURIDINE

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ABSTRACT *Haemophilus influenzae* was labeled with thymidine-^{3}H (dThd), then grown in the presence of 5-bromodeoxyuridine (BrdUrd), and then irradiated with 313 nm light (a wavelength that selectively photolyzes DNA containing 5-bromouracil [BrUra]). Irradiation with 313 nm light induced breaks in the ^{3}H-labeled strands in cells grown with BrdUrd at a much higher frequency than in ^{14}C-labeled DNA of cells not exposed to BrdUrd. Breakage of the ^{3}H-labeled strands was about 0.6% as efficient as that of fully BrUra-substituted DNA. During growth in the presence of BrdUrd, susceptibility to 313 nm-induced breakage of the ^{3}H-labeled DNA strands increased, reaching a maximum in about one generation, and it decreased to zero during subsequent growth for one generation in medium containing dThd instead of BrdUrd. Heat denaturation of DNA extracted from dThd-^{3}H-labeled cells grown in the presence of BrdUrd eliminated 313 nm-induced breakage of the ^{3}H-labeled strands. It is concluded that breakage of the ^{3}H-labeled DNA strands resulted from reaction with photoproducts in the base-paired, BrUra-containing strands, rather than from photolysis of BrdUrd incorporated into parental strands. It may be possible to utilize the phenomenon of interstrand breakage in physical studies of DNA replication.

INTRODUCTION

It has been shown that ultraviolet (UV) irradiation[1] induces single strand breaks in DNA containing BrUra (Lion, 1966; Hutchinson and Köhnlein, 1967). The preferential breakage of BrUra-containing DNA compared with unsubstituted DNA is much greater at 313 nm than at shorter wavelengths (R. B. Setlow, W. L. Carrier, and R. D. Ley, manuscript in preparation), and it has been utilized to determine

[1] Although there is some evidence that the single strand breaks are produced directly upon irradiation (Hutchinson and Hales, 1970; Lion, 1970 *b*), other evidence supports the view that most (Hewitt and Marburger, 1971) and perhaps all (Lion, 1970 *a*) of the breaks represent alkali labile bonds produced by the irradiation.

the extent of DNA repair after UV irradiation of mammalian cells (Regan et al., 1971) and the length of repaired regions in the DNA of UV-irradiated bacteria (Ley and Setlow, 1972). The phenomenon has also been used to study postreplication repair of DNA in UV-irradiated mouse cells (Lehmann, 1972). Preliminary work with hybrid *Escherichia coli* DNA (R. B. Setlow and W. L. Carrier, manuscript in preparation) suggests that 313 nm irradiation may induce a very small number of breaks in unsubstituted DNA strands that are base-paired with BrUra-substituted strands, but this effect does not seem to be great enough to interfere with measurement of the length of repaired regions in the DNA of UV-irradiated *E. coli*.

In an attempt to use 313 nm-induced strand breakage to detect repair replication in *H. influenzae*, it was found that 313 nm irradiation of untreated cells grown in the presence of BrdUrd after labeling with dThd-^{3}H resulted in a significant amount of breakage of the ^{3}H-labeled DNA strands. The breakage could have resulted either from insertion of BrUra-containing regions of DNA into parental DNA strands during replication in the presence of BrdUrd (by resynthesis or genetic recombination at certain regions of the DNA) or from reaction between the ^{3}H-labeled strands and UV-induced lesions in the opposite, BrUra-containing strands. This paper reports evidence favoring the latter possibility.

MATERIALS AND METHODS

Bacteria and Growth Conditions

The strain of *H. influenzae* used in most of this work was Rd thy$^-$2, a thymine-requiring spontaneous mutant isolated from wild-type strain Rd by the method of Chevallier and Greth (1971). The recombination-deficient strains, DB117 and Rd(DB117)$^{rec-}$, have been previously described (Setlow et al., 1968; Boling and Setlow, 1969; Beattie and Setlow, 1971). Cells were grown either in 3.5% brain heart infusion (BHI) (Difco Labs, Detroit, Mich.) supplemented with 10 μg/ml hemin and 3 μg/ml nicotinamide adenine dinucleotide, or in medium MI$_o$-Cit (Herriott et al., 1970) containing 5 μg/ml dThd or BrdUrd. Cells were grown at 37°C with aeration.

Radioactive Labeling of Bacteria

Cells were labeled during exponential growth with 50 μCi/ml dThd-methyl-^{3}H (16 Ci/mmole, Schwarz Bio Research Inc., Orangeburg, N.Y.), 1 μCi/ml dThd-2-^{14}C (53 mCi/mmole, Schwarz Bio Research Inc.), or 0.2 μCi/ml BrdUrd-2-^{14}C (48.8 mCi/mmole, Schwarz Bio Research Inc.). When cells were labeled in BHI, 250 μg/ml inosine was added. When cells were labeled with dThd-^{3}H in medium MI$_o$-Cit, 5 μg/ml unlabeled dThd was added. When labeling was done after growth in the presence of BrdUrd, the cells were centrifuged and washed with growth medium before addition of radioactivity.

Incubation of Cells with BrdUrd

When radioactive labeling preceded growth in the presence of BrdUrd, the cells were centrifuged, washed with growth medium, resuspended in growth medium, and grown for about one-half generation (20 min) before addition of BrdUrd. When cells were grown in BHI,

100 μg/ml BrdUrd was added, and growth continued normally for about three generations. When cells were grown in MI_c-Cit, 5 μg/ml BrdUrd was added, and growth continued normally for about two generations. Incubation of cells with BrdUrd was done under yellow lights.

Irradiation with 313 nm Light

Before 313 nm irradiation, cells were centrifuged, washed with ice-cold buffer containing 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, and 0.1% NH_4Cl, and resuspended in the same buffer at 5×10^8 cells/ml (OD 675 = 0.20). Just before irradiation, equal volumes of dTHd-^{14}C-labeled (control) and dThd-^{3}H-labeled (BrdUrd-treated) cells were mixed, and 0.25 ml cells were placed in an ice-cooled, quartz microcuvette with a 1-cm light path. Irradiation was carried out with a Hilger quartz prism monochromator (Hilger & Watts Inc., Morton Grove, Ill.) illuminated by a Philips 1000 w high pressure mercury arc lamp (Philips Electronic Instruments, Mount Vernon, N.Y.), with a band width of 7 nm. The 313 nm light was passed through a thin Mylar film (10% transmission at 308 nm, 1% at 306 nm) to eliminate scattered shorter wavelengths. The average intensities, through the samples were determined using a bolometer (Yellow Springs Instrument Co., Yellow Springs, Ohio) calibrated against a standard lamp (National Bureau of Standards) and were corrected for absorption by the cell suspension or DNA solutions.

Determination of Average Molecular Weights of DNA and Calculation of Number of Strand Breaks

To determine the single strand molecular weights of irradiated-extracted DNA or of DNA in irradiated cells, 0.10 ml of DNA solution or cell suspension was gently layered onto 5-ml linear 5–20% alkaline sucrose gradients (McGrath and Williams, 1966) containing 0.5 M NaCl and 0.01 M ethylenediaminetetraacetate (EDTA) (pH 12) after 0.10 ml 1 N NaOH had first been layered onto the gradients. The gradients were made ½ × 2 inch polyallomer tubes (Beckman Instruments, Inc., Fullerton, Calif.). After 15 min at room temperature for lysis of cells on the gradients, centrifugation was carried out at 30,000 rpm at 13°C in a SW-50.1 rotor and Beckman model L ultracentrifuge (Beckman Instruments, Inc.). DNA liberated from cells lysed on the gradients was centrifuged for 90 or 100 min, and extracted DNA was centrifuged for 4 or 5 hr. The distribution of radioactivity in the gradients was determined by puncturing the bottom of the tubes and collecting approximately 30 10-drop fractions on Whatman No. 17 paper strips (Carrier and Setlow, 1971). After the strips were washed once with 5% trichloroacetic acid (TCA) and twice with 95% ethanol and dried, they were cut into sections and the TCA-insoluble radioactivity was counted in a toluene-2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiopene (BBOT) scintillator using a Packard Tri-Carb liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, Ill.). The data were put into a computer which calculated number average (M_n) and weight average (M_w) molecular weights, using a computer program written by M. L. Randolph and J. E. Donnellan. The number of 313 nm-induced strand breaks per single-stranded genome was calculated using number average molecular weights and a molecular weight of 8×10^8 daltons for the single-stranded *H. influenzae* genome (Gillis et al., 1970). When dThd-^{3}H-labeled, BrdUrd-treated cells were mixed with untreated dThd-^{14}C-labeled cells before 313 nm irradiation, the number of UV-induced strand breaks per 8×10^8 daltons of ^{3}H-labeled DNA strands was calculated using the equation

$$\text{strand breaks} = 8 \times 10^8 \left[\frac{1}{M_n(^3\text{H})} - \frac{1}{M_n(^{14}\text{C})} \right].$$

Extraction of DNA from Bacteria

Cells were washed with a solution of 0.15 M NaCl and 0.05 M sodium citrate (SSC), and resuspended in SSC at 10^9–10^{10} cells/ml. After addition of 50 μg/ml lysozyme, cells were incubated at 37°C for 30 min, and then sodium dodecylsulfate (1% final concentration) and sodium perchlorate (1 M final concentration) were added. The lysate was shaken gently with an equal volume of chloroform:isoamyl alcohol (24:1) at room temperature for 30 min and centrifuged at 2500 rpm for 20 min, and the aqueous layer was removed. The DNA was then precipitated by adding 2 vol of 95% ethanol and dissolved in SSC. When the DNA contained BrUra, the extraction was carried out under yellow lights.

RESULTS AND DISCUSSION

Breakage of Parental DNA Strands Induced by 313 nm Light after Replication in the Presence of BrdUrd

When dThd-^{3}H-labeled *H. influenzae* cells are grown in the presence of BrdUrd and then irradiated with 313 nm light, the single strand molecular weight of the ^{3}H-labeled DNA is decreased. Fig. 1 shows the results of a typical experiment in which dThd-^{3}H-labeled cells were grown in nonradioactive BHI for one-half generation, then for two generations in the presence of 100 μg/ml BrdUrd, irradiated at 313 nm, lysed on alkaline sucrose gradients, and centrifuged. At 10^5 ergs/mm^2 (Fig. 1 A) the number average molecular weight of the ^{3}H-labeled DNA strands was 12.9×10^6 daltons, compared with 43.9×10^6 daltons for dThd-^{14}C-labeled cells not exposed to BrdUrd. This difference corresponds to 43 radiation-induced strand breaks per single-stranded genome, based on a single strand molecular weight of 8×10^8 for *H. influenzae* DNA in vivo (Gillis et al., 1970). Figure 1 B shows that breakage of the ^{3}H-labeled DNA strands is a linear function of 313 nm fluence.

Dependence of Strand Breakage on Duration of Growth in the Presence of BrdUrd

Fig. 2 (solid curve) shows that 313 nm-induced breakage of ^{3}H-labeled DNA strands increased as a function of growth time with BrdUrd, leveling off at about 30 min. This time is close to the doubling time for *H. influenzae* under these growth conditions. The dashed curve shows 313 nm-induced breakage of the ^{3}H-labeled DNA strands after cells were grown with BrdUrd for 40 min, then centrifuged, washed, and resuspended in medium containing dThd and no BrdUrd. During growth in the absence of BrdUrd, breakage of the ^{3}H-labeled DNA strands decreased and returned essentially to zero in about 40 min. Again, this is close to one doubling time.[2]

These data are consistent with the concept that 313 nm-induced breakage of the ^{3}H-labeled DNA strands involves BrUra photoproducts in the opposite (base-paired) DNA strands, since maximum breakage occurred after one complete round

[2] The cells grew slightly less rapidly during this period than during the first 40 min with BrdUrd.

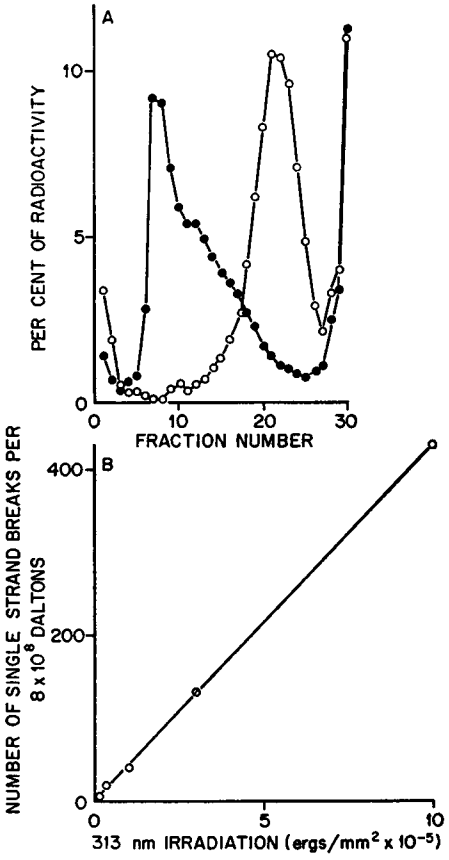

FIGURE 1

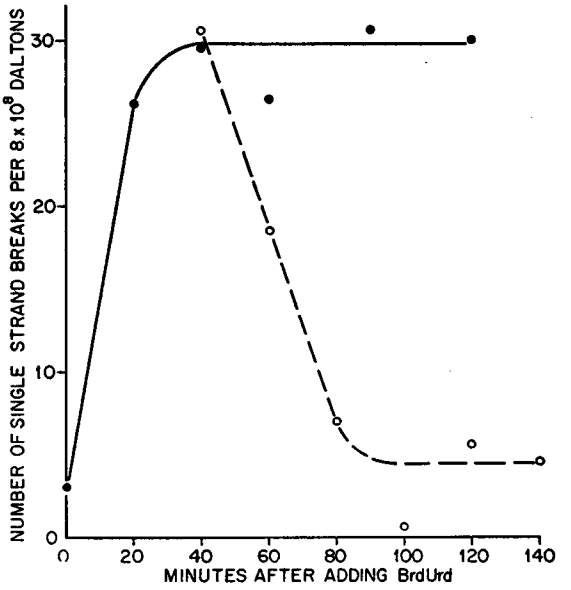

FIGURE 2

FIGURE 1 (A) Alkaline sucrose sedimentation profiles of DNA from prelabeled *H. influenzae* grown with or without BrdUrd, then irradiated with 10^5 ergs/mm² 313 nm light. Filled circles, dThd-^{14}C-labeled cells not exposed to BrdUrd; open circles, dThd-^{3}H-labeled cells washed and grown for 20 min without BrdUrd, then grown in BHI for 90 min with 100 μg/ml BrdUrd. Gradients were centrifuged at 30,000 rpm for 90 min. (B) Strand breakage of dThd-^{3}H-labeled *H. influenzae* DNA induced by 313 nm light after growth in BHI for 90 min with 100 μg/ml BrdUrd, plotted as a function of 313 nm fluence. Gradients were centrifuged at 30,000 rpm for 90 min.

FIGURE 2 Strand breakage induced by 313 nm light in dThd-^{3}H-labeled DNA from prelabeled *H. influenzae* grown for various times with 100 μg/ml BrdUrd. Filled circles, strand breakage after addition of BrdUrd at time zero; open circles, strand breakage after washing cells 40 min after adding BrdUrd, then growing for various times in BHI without BrdUrd. Cells were irradiated with 5×10^4 ergs/mm² at 313 nm, and gradients were centrifuged at 30,000 rpm for 100 min.

of replication (at which time the maximum number of BrUra residues were base-paired with the ^{3}H-labeled strands), and a subsequent round of replication in the absence of BrdUrd eliminated 313 nm-induced breakage of the ^{3}H-labeled strands. Although the data of Fig. 2 do not eliminate the possibility that certain regions of

parental DNA strands are constantly being replaced by repair synthesis or recombination with newly synthesized strands, the data do put severe restrictions on such a hypothesis, since the replacements would have to occur once every generation, and at precisely the same regions each time.

Evidence against 313 nm-Induced Breakage Resulting from Replacement of Certain Regions in Parental DNA Strands

Fig. 3 shows the results of an experiment in which cells were grown for two generations with BrdUrd, washed, grown for one-third generation without BrdUrd, then labeled with dThd-^{3}H for two-thirds generation before irradiation at 313 nm. The majority of the dThd-^{3}H should have been incorporated into DNA strands opposite BrUra-containing parental strands. At 5×10^4 ergs/mm^2, the number average molecular weight of the dThd-^{3}H-labeled DNA strands was 24×10^6, compared with 78×10^6 for dThd-^{14}C-labeled DNA from cells not exposed to BrdUrd. Based on a molecular weight of 8×10^8 for single strands of the *H. influenzae* genome, this corresponds to 24 strand breaks per ^{3}H-labeled DNA strand. This is comparable with the amount of breakage obtained when cells were dThd-^{3}H-labeled first, then grown with BrdUrd for at least one generation before irradiation with 5×10^4 ergs/mm^2 313 nm light (Fig. 2). These data are strong evidence against dependence of 313 nm-induced breakage of the ^{3}H-labeled strands on breakdown and resynthesis at certain regions of the genome, since under the conditions of this experiment any resynthesized regions would contain dThd and not BrdUrd, and should not lead to strand breakage upon 313 nm irradiation.

Breakage of the ^{3}H-labeled DNA strands induced by 313 nm light after growth with BrdUrd was observed not only in wild-type *H. influenzae*, but also in two different recombination-deficient strains, DB117 and Rd(DB117)$^{rec-}$. Both of these strains fail to recombine temperature-sensitive phages and exhibit extremely low transformation frequencies (Boling and Setlow, 1969; Beattie and Setlow, 1971). The results with the rec$^-$ strains argue against an explanation for strand breakage based on insertion of BrUra-containing sections of DNA into parental strands by recombination between parental and newly synthesized strands.

To provide further evidence against dependence of 313 nm-induced breakage of the ^{3}H-labeled strands on insertion of BrUra-containing sections of DNA into parental strands during growth with BrdUrd, the following experiment was performed. dThd-^{3}H-labeled cells were grown for one generation with BrdUrd, then washed and mixed with ^{14}C-labeled control cells, and the DNA was extracted and irradiated at 313 nm. As seen in Fig. 4 A, 6×10^5 ergs/mm^2 resulted in a large reduction in the molecular weight of the ^{3}H-labeled strands ($M_w = 2.3 \times 10^6$) compared with the ^{14}C-labeled control DNA ($M_w = 7.8 \times 10^6$). When the DNA was heat-denatured before irradiation (Fig. 4 B), there was very little difference between

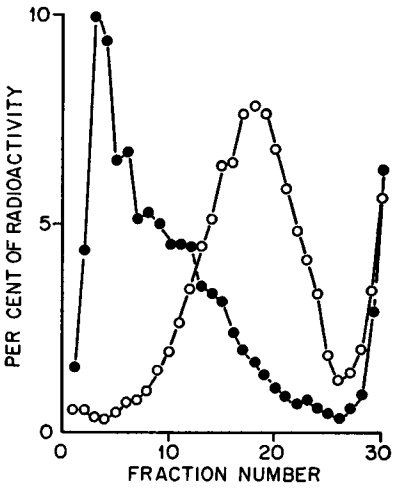

FIGURE 3

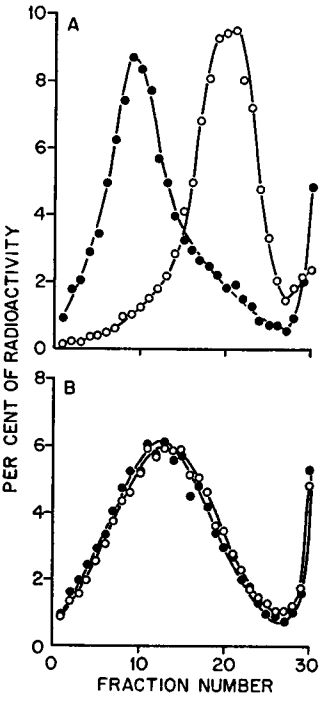

FIGURE 4

FIGURE 3 Alkaline sucrose sedimentation profiles of DNA from *H. influenzae* grown with or without BrdUrd, then labeled with dThd-^{3}H or dThd-^{14}C and irradiated with 4 × 10^{5} ergs/mm^{2} 313 nm light. Filled circles, cells not previously exposed to BrdUrd and labeled with dThd-^{14}C for 120 min in BHI; open circles, cells grown in BHI containing 100 μg/ml BrdUrd for 90 min, then washed and grown in BHI for 15 min, then labeled with dThd-^{3}H for 30 min in BHI. Gradients were centrifuged at 30,000 rpm for 100 min.

FIGURE 4 (A) Alkaline sucrose sedimentation profiles of 313 nm-irradiated DNA extracted from prelabeled *H. influenzae* grown in MI_{o}-Cit with or without 5 μg/ml BrdUrd; 313 nm fluence = 6 × 10^{5} ergs/mm^{2}. Filled circles, cells labeled with dThd-^{14}C in MI_{o}-Cit for 120 min, not exposed to BrdUrd; open circles, cells labeled with dThd-^{3}H in MI_{o}-Cit for 120 min, then grown for 45 min in MI_{o}-Cit containing 5 μg/ml BrdUrd. Gradients were centrifuged at 30,000 rpm for 300 min. (B) Same as A, except the solution of DNA was boiled for 10 min, then plunged into ice, just before irradiation.

the molecular weight of ^{3}H-labeled and ^{14}C-labeled DNA strands.[3] The small difference seen in Fig. 4 B is probably not significant, since it was also seen in the absence of 313 nm irradiation. These data strongly suggest that 313 nm-induced breakage of the unsubstituted (^{3}H-labeled) DNA strands seen in these experiments depends on the presence of BrUra in strands base-paired with the ^{3}H-labeled strands, rather than the presence of BrUra in the ^{3}H-labeled strands.

[3] It has been shown by R. B. Setlow and W. L. Carrier (manuscript in preparation) that denaturation of BrUra-containing *E. coli* DNA does not decrease the 313 nm-induced strand breakage of that DNA.

Origin of 313 nm-Induced Breaks in Unsubstituted DNA Strands Base-Paired with BrUra-Containing Strands

Alterations in BrUra-containing DNA associated with UV irradiation include debromination and formation of uracilyl radicals (Rupp and Prusoff, 1965; Ishihara and Wang, 1966), damaged sugar residues[4] (Hotz and Reuschl, 1967; Köhnlein and Hutchinson, 1969), and breakage of *N*-glycosyl bonds (Lion, 1970 *b*). The radical scavenger, cysteamine, protects BrUra-substituted DNA from strand breakage induced by UV irradiation (Lion, 1970 *a*) and protects the DNA from damage to sugar residues (Hotz and Reuschl, 1967) and from breakage of glycosidic bonds (Lion, 1970 *b*). As seen in Fig. 5, 313 nm-induced breakage of dThd-^{3}H-labeled DNA strands base-paired with BrUra-containing strands is also greatly reduced in the presence of cysteamine.[5] The data of Fig. 5 suggest that a UV-induced radical is involved in breakage of unsubstituted DNA strands base-paired with BrUra-containing strands. Different types of radicals, however, may be involved in the breakage of the unsubstituted strands and the breakage of the BrUra-containing strands.

From data such as those shown in Figs. 1 B and 5, it is possible to estimate the number of strand breaks induced per BrUra residue per erg·mm^{-2}. Based on four different determinations, the efficiency for breakage of unsubstituted strands base-paired with BrUra-substituted strands is about 6.6×10^{-10} strand breaks per BrUra residue per erg·mm^{-2}. The efficiency varied by a factor of two in different experiments, which is not surprising, considering the many possible sources of error (Ley and Setlow, 1972). Based on similar experiments, the breakage efficiency of fully BrUra-substituted strands of *H. influenzae* DNA is about 1.2×10^{-7} strand breaks per BrUra residue per erg·mm^{-2}. Thus, 313 nm-induced breakage of unsubstituted strands base-paired with BrUra-substituted strands appears to occur about 0.6% as frequently as breakage of the BrUra-substituted strands.

Under experimental conditions very similar to those used in this work, Ley and Setlow (1972) found that growth of dThd-^{3}H-labeled *E. coli* cells with BrdUrd led to very little, if any, increased sensitivity of the ^{3}H-labeled DNA to 313 nm-induced single strand breakage, compared with cells not exposed to BrdUrd. This seems to conflict with the results with *H. influenzae*, but this disparity may be caused by differences in the structure of the DNA (and therefore the photochemistry) in the two organisms, which may be reflected by the base compositions (50% G + C for *E. coli* vs. 39% G + C for *H. influenzae*). It has been shown that DNA structure depends both on base composition (Bram, 1971) and on nucleotide sequence (Wells et al., 1970). The conformation of BrUra-containing DNA at certain regions of the

[4] As pointed out by Köhnlein and Hutchinson (1969), attack by uracilyl radicals on sugar residues is feasible, since in the B configuration of BrUra-containing DNA, each Br atom is in van der Waals contact with the sugar in the nucleotide 5′ to the BrdUrd nucleotide.

[5] The absorbance of 100 mM cysteamine is insignificant at 313 nm.

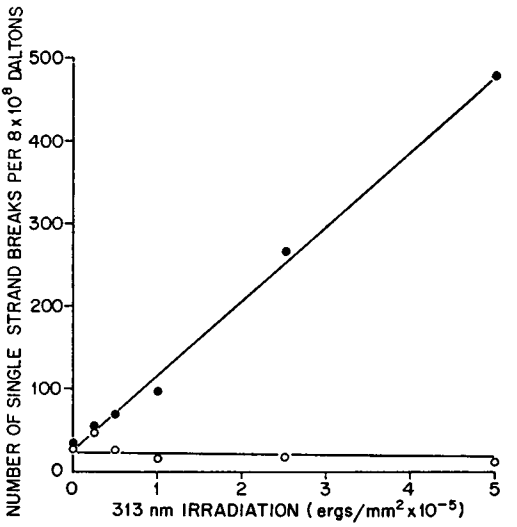

FIGURE 5 Strand breakage induced by 313 nm light in dThd-^{3}H-labeled DNA extracted from prelabeled *H. influenzae* cells grown for 45 min in MI_c-Cit containing 5 μg/ml BrdUrd. The DNA was irradiated at various 313 nm fluences in the presence (open circles) or absence (filled circles) of 100 mM cysteamine. Gradients were centrifuged at 30,000 rpm for 240 min.

H. influenzae genome may allow the usual UV-induced radicals to react with sites on the opposite, unsubstituted strand or may allow formation of unique photoproducts that can react with the opposite strand of the double helix. There may be very few regions in the *E. coli* chromosome that have the proper conformation for interstrand breakage.

Since in *H. influenzae* 313 nm irradiation results in breakage of unsubstituted DNA strands base-paired with BrUra-containing strands, measurement of photolysis of BrUra-containing DNA may not be a particularly sensitive method for detecting repair synthesis in this organism. It may be possible, however, to utilize the phenomenon of interstrand breakage in the study of DNA replication. For example, irradiation of ^{3}H-labeled cells after limited replication in the presence of BrdUrd should result in preferential breakage of regions of the parental DNA strands which have replicated. By analyzing the irradiated material by alkaline sucrose gradient centrifugation, it should be possible to estimate the number of growing points in the DNA under various conditions.

The author thanks Doctors R. B. Setlow, J. K. Setlow, and M. L. Randolph for helpful suggestions and the use of laboratory facilities.

This research was sponsored by the U.S. Atomic Energy Commission under contract with Union Carbide Corporation.

Received for publication 31 May 1972.

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