STRAND BREAKS AND ALKALI-LABILE BONDS INDUCED BY ULTRAVIOLET LIGHT IN DNA WITH 5-BROMOURACIL IN VIVO

FRANK KRASIN AND FRANKLIN HUTCHINSON, *Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06520 U. S. A.*

ABSTRACT Supercircular λ phage DNA with 10 bromouracils/100 thymine bases, irradiated with 313 nm light in Tris buffer and sedimented on alkaline and neutral gradients, showed 4.6 alkali-labile bonds per true single-strand break, in agreement with Hewitt and Marburger (1975 *Photochem. Photobiol.* **21**:413). The same DNA irradiated in *Escherichia coli* host cells showed about the same number of breaks in alkaline gradients for equal fluence, but only 0.5 alkali-labile bond per true break. Similarly, *E. coli* DNA with bromouracil irradiated in the cells showed only 10–20% more breaks when denatured with 0.1 M NaOH than under neutral conditions with 9 M sodium perchlorate at 50°C.

These results show that true single-strand breaks occur more frequently than alkali-labile bonds after ultraviolet irradiation of DNA containing bromouracil in cells.

INTRODUCTION

Experiments on the production of double-strand breaks by ultraviolet irradiation of intracellular DNA containing bromouracil are described in the preceding paper (Krasin and Hutchinson, 1978). The results are not easy to reconcile in detail with results previously reported on the photochemistry of bromouracil in DNA. In particular, it is shown in the previous paper that the number of double-strand breaks is roughly equal to the numbers from the literature of breaks in a strand containing only thymine paired with a strand containing bromouracil. This suggests that a break in the strand complementary to that containing the photochemically decomposed bromouracil usually is part of a double-strand break. However, Hewitt and Marburger (1975) have shown quite elegantly that four out of five breaks (as assayed in alkaline gradients) in bromouracil-DNA supercircles irradiated in Tris buffer with ultraviolet light are actually alkali-labile bonds. Thus, there are two possibilities: (*a*) single-strand breaks in the strand complementary to that with the affected bromouracil do not occur opposite an alkali-labile bond, but only near a true single-strand break; (*b*) the ratio of alkali-labile bonds to true single-strand breaks is low for DNA in cells where the occurrence of double-strand breaks was measured.

This paper presents the results of experiments in which bromouracil-DNA supercircles were irradiated with ultraviolet light inside *E. coli* cells, and also under the conditions used by Hewitt and Marburger. In buffer, we reproduced Hewitt and

Marburger's results; in cells the ratio of alkali-labile bonds to true breaks dropped by an order of magnitude. The small fraction of alkali-labile bonds in intracellular DNA after irradiation was confirmed in *E. coli* bromouracil-DNA denatured either by alkali or by gentle heating in sodium perchlorate. Thus, the second possibility, a reduction in the ratio of alkali-labile bonds to true breaks for DNA in cells, is correct.

MATERIALS AND METHODS

Preparation of λ Phage DNA Containing Bromouracil

Phage λc71, *E. coli* CR34 and *E. coli* CR34 (λ *ind*$^-$) were obtained from Dr. Era Cassuto. *E. coli* CR34 cells were infected by phage λc71 at a multiplicity of 5 per cell in medium containing *methyl*-[^{3}H]thymine (34 μCi/ml, 5 μg/ml) and 5-[2-^{14}C]bromouracil (0.4 μCi/ml, 5 μg/ml).

Covalently closed supercircular DNA was prepared as described by Ross and Howard-Flanders (1977). Purified phage obtained from the lysate were used to infect *E. coli* CR34 (λ *ind*$^-$) at about 15 per cell. After 30 min incubation at 37°C and cell lysis with lysozyme (200 μg/ml) and Sarcosyl (0.2%) at ice temperature, about 50% of the labeled DNA was in the form of covalently closed circles as determined by neutral sucrose sedimentation.

313 nm Irradiation

Irradiations were carried out with light from a 500 W medium pressure mercury lamp passed through an interference filter peaked at 313 nm (see Krasin and Hutchinson, 1978, for details). All cells were irradiated in M9 buffer (19.7 mM NH_4Cl, 43.7 mM Na_2HPO_4, 23.2 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 0.1 μg thiamine/ml) at ice temperature and with stirring. Other details are given in the figure captions.

Gradient Sedimentation

Breaks in supercircular λ DNA were measured in neutral 5–20% (wt/vol) sucrose (with 1 M NaCl) and alkaline 5–20% (wt/vol) sucrose (with 0.7 M NaCl, 0.3 M NaOH) as described by Hewitt and Marburger (1975). Sedimentation was for 35 or 60 min (neutral) and 35 min (alkaline) at 45,000 rpm in an SW50.1 rotor at 20°C.

Alkali-labile bonds and true single-strand breaks produced in irradiated *E. coli* DNA containing bromouracil were determined by sedimenting the denatured DNA in neutral sucrose (5–20% wt/vol) gradients with 0.5 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.6, in an SW50.1 rotor at 20°C.

RESULTS

Alkali-Labile Bonds in Supercircular λ DNA

The numbers of breaks induced by 313 nm light in supercircular λ DNA with about 10 bromouracil residues per 100 thymines are plotted in Fig. 1. For each condition of irradiation, the number of breaks n was calculated from the fraction f of radioactivity sedimenting as supercircles;

$$f = f_0 \exp(-n) \tag{1}$$

f_0 is the fraction of supercircles in unirradiated DNA.

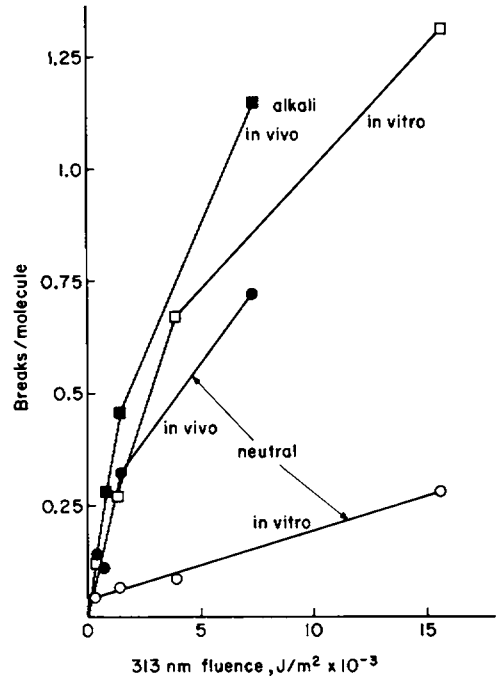

FIGURE 1 The number of breaks in supercircular λ phage DNA containing bromouracil produced by 313 nm radiation under various conditions. *E. coli* CR34 (λ *ind*⁻) cells were infected with λ c71 phage whose DNA was radioactively labeled and contained about 10 bromouracil/100 thymine residues. For in vivo irradiation, the infected cells were resuspended in M9 buffer at 2×10^8/ml and irradiated for various times with 313 nm light at ice temperature. The cells were lysed, and lysates sedimented on both neutral and alkaline sucrose gradients. The numbers of breaks introduced by irradiation were calculated from the radioactivity in the fast sedimenting supercircular fraction, f, using Eq. 1 in the text, and f_0 (supercircular fraction from unirradiated cells), equal to 0.54 for neutral gradients and 0.44 for alkaline gradients. For in vitro irradiations, infected cells from the same batch were lysed, and the lysate extensively dialyzed against the buffer used by Hewitt and Marburger (1975), 0.01 Tris, 0.001 M EDTA, 0.15 M NaCl, pH 8.1. The supercircular DNA was irradiated with 313 nm light at ice temperature, then sedimented on neutral and alkaline gradients. The number of breaks introduced by irradiation was again calculated with Eq. 1, with $f_0 = 0.52$ for neutral gradients and 0.42 for alkaline gradients. Supercircles with less bromouracil than most of the population could account for the bending over of the curves. At these higher numbers of breaks per molecule, only about 10% of the activity sedimented as supercircles; the presence of a low radioactivity "background" at the position of the supercircles also could account for the bending over of the curves.

For irradiation in buffer (open symbols in Fig. 1) the DNA showed many more breaks on alkaline gradients than when sedimented on neutral gradients. This confirmed the presence of many alkali-labile bonds, in agreement with Hewitt and Marburger (1975).

When the DNA was irradiated in cells (closed symbols in Fig. 1), the number of breaks measured on alkaline gradients probably did not differ significantly from the

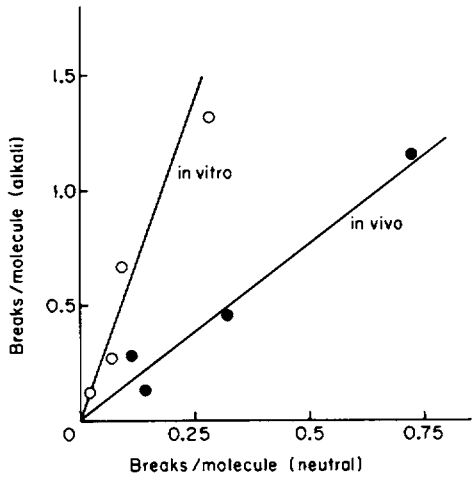

FIGURE 2

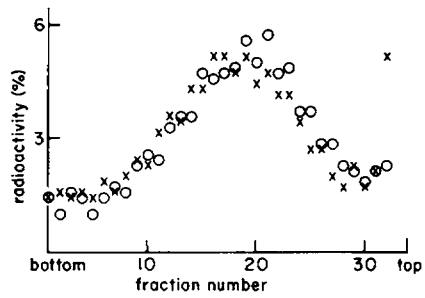

FIGURE 3

FIGURE 2 A replot of the data in Fig. 1, the number of breaks in alkali against the number in neutral gradients for the same DNA sample.

FIGURE 3 Neutral gradient sedimentation of *E. coli* DNA containing bromouracil after irradiation and denaturation with either alkali or gentle heating in perchlorate. *E. coli* AB2497 wild-type cells were grown in K medium containing both thymine and bromouracil so that the DNA contained about 14 bromouracil residues/100 thymines. The cells were resuspended in M 9 buffer at $1\text{-}2 \times 10^9$/ml, irradiated with 313 nm light at ice temperature (with stirring), and lysed. One portion of the lysate was diluted 10-fold with 10 M sodium perchlorate, 0.017 M EDTA, pH 7.0, then the DNA denatured by heating to 50°C for 10 min (Geiduschek, 1962) and quickly chilled. In another portion of the same lysate, the DNA was denatured with 0.1 M NaOH for 5 min at room temperature, before addition of HCl to neutrality. Each portion was adjusted to 0.45 M Na^+, then sedimented on a neutral sucrose gradient (0.5 M NaCl) for 16 h at 20,000 rpm in an SW50.1 rotor at 20°C. (x) Denatured with perchlorate at 50°C. (o) Denatured with 0.1 M NaOH.

number so determined in DNA irradiated in buffer. The important difference was the large increase in breaks measured in neutral gradients, which showed the presence of relatively few alkali-labile bonds.

The data in Fig. 1 are replotted in Fig. 2, with the number of breaks (alkali) as a function of the breaks (neutral) in the same irradiated DNA sample. This shows 5.6 breaks (alkali) per break (neutral) for DNA irradiated in buffer, compared to a ratio of 5 found by Hewitt and Marburger (1975). For DNA irradiated in cells, the ratio is 1.5 breaks (alkali) per break (neutral), or roughly 0.5 alkali-labile breaks per "true" single-strand break.

Alkali-Labile Bonds in E. coli DNA

Fig. 3 compares the neutral gradient sedimentation of single-strand bromouracil-DNA from *E. coli* cells irradiated with 313 nm light after denaturation by heating to 50°C in 9 M sodium perchlorate, and after denaturation by 0.1 M NaOH. The sedimentation patterns are seen to be very similar, showing that alkali treatment produces only

a few more breaks than gentle heating in perchlorate. Unirradiated DNA treated in the same way sedimented six- to sevenfold faster, showing a molecular weight over 100 times larger. The dependence on mass M of the sedimentation coefficient of single-strand DNA in neutral gradients is strongly influenced by salt concentration (Studier, 1965) and is not accurately known. Assuming a dependence of M^{α}, with α between 0.38 and 0.50, the sedimentation profiles in Fig. 3 give number-average molecular weights of 0.07–0.2 $\times$ 10^6 daltons, assuming a mass of 15 $\times$ 10^6 daltons for the reference single-strand λ phage DNA. For all values of α the alkali-denatured DNA had a mass 10–20% lower than did the perchlorate-heat-denatured DNA. Therefore, 0.1–0.2 bonds/break opened in alkali that did not open during thermal melting at 50°C in the presence of perchlorate.

The following control experiment demonstrated that the DNA did denature at 50°C with perchlorate. DNA from unirradiated cells was gently sheared. Its number-average molecular weight was 27 $\times$ 10^6 daltons, compared to reference λ phage DNA (30 $\times$ 10^6 daltons), assuming that sedimentation in neutral sucrose varies as $M^{0.38}$. This sheared DNA was denatured either with perchlorate or with alkali, exactly as the irradiated samples, then sedimented on identical neutral gradients. The DNAs denatured in the two different ways sedimented at the same rate, and at a rate 2.0 greater than the native duplex form (data not shown). Rosenberg and Studier (1969) found the denatured T7 DNA sedimented 2.4-fold faster than did the native duplex form on neutral gradients of essentially the same composition as ours.

The Number of Single-Strand Breaks and Uracil Residues

A cross section σ_S for the production of DNA single-strand breaks (measured in alkali) can be defined.

$$\sigma_S = \frac{\text{breaks/unit length}}{(\text{fluence, J/m}^2)(\text{bromouracil bases/unit length})}. \qquad (2)$$

E. coli AB2497 wild-type cells were grown as described in Materials and Methods, in medium containing 3.6 μg/ml 5-[6-^{3}H]bromouracil (40 μCi/ml) and 5.5 μg/ml [*methyl*-^{14}C]thymine (2 μCi/ml). The cells were irradiated with 20–57 $\times$ 10^3 J/m^2 of 313 nm light and lysed, and the lysates were centrifuged on alkaline sucrose gradients. ^{3}H-labeled T2 DNA sedimented in identical gradients was used for calibration purposes. The number-average molecular weights were calculated from the sedimentation profiles by fitting theoretical curves (Levin and Hutchinson, 1973), assuming the mass of single-strand T2 DNA is 55 $\times$ 10^6 daltons, and sedimentation of DNA of mass M in alkaline gradients depends on $M^{0.40}$.

The number of bromouracil bases per unit length was calculated by determining the ratio of ^{3}H activity to ^{14}C activity in both the DNA and in the medium in which the cells were grown (which had a known bromouracil/thymine molecular ratio), allowing for the various corrections discussed by Rydberg (1977). In this DNA, 6 thymines/100 had been replaced by bromouracil.

Using this figure and the various measurements of single-strand breaks, Eq. 2 gave

$\sigma_S = 0.45 \times 10^{-6}$ m^2/J. Consideration of the errors involved would suggest that this is correct to about ±25%.

To determine the production of uracil, *E. coli* AB2497 cells were grown overnight in K medium with 5 μg/ml thymine, 0.2 μg/ml 5-[6-^{3}H]bromouracil (20 μCi/ml, 20 Ci/mmol), and 50 μg/ml uracil (the last to depress incorporation of [6-^{3}H]uracil, a radiolysis product in the radioactive bromouracil preparation). The DNA was extracted with phenol, treated vigorously with RNase, reextracted with phenol, and dialyzed extensively against 0.01 M Tris buffer, pH 7.0. Less than 1% of the thymine was replaced by bromouracil. Samples were irradiated with 313 nm light to several fluences between 0 and 6×10^4 J/m^2. Samples were resuspended in trifluoroacetic acid, hydrolyzed 90 min at 127°C in sealed glass tubes, then chromatographed on Whatman no. 1 paper (Whatman Inc., Clifton, N.J.) in acetic acid:water:butanol:: 12:30:80 for 16 h. The paper was cut in strips and the radioactivity in each strip determined. The only significant peak, aside from that for bromouracil ($R_f = 0.64$), was that for uracil ($R_f = 0.50$).

A cross section σ_U for the production of uracil can be defined.

$$\sigma_U = \frac{U/(U + B) - [U/(U + B)]_{\text{control}}}{\text{fluence, J/m}^2}. \tag{3}$$

Here, U is the radioactivity in the uracil peak, and B the radioactivity in the bromouracil peak. The average of several determinations was $\sigma_U = 0.76 \times 10^{-6}$ M^2/J, with a probable error of the mean of about ±10%.

The absolute value of these cross sections is significant only within a factor of 2 or so. The cross section of bromouracil is a steep function of the wavelength in the vicinity of 313 nm (review: Hutchinson, 1973), and therefore the cross section will depend critically on precisely how the irradiating beam is produced. The ratio σ_U/σ_S is more significant, and shows that about 1.5 (±35%) uracils are produced per single-strand break (in alkali). An assumption made here, that uracil production will be the same in DNA within cells and in solution, is justified by several observations that uracil production is independent of the solution surrounding the DNA during irradiation (review: Hutchinson and Köhnlein, 1978).

It is known that -SH compounds suppress the formation of single-strand breaks, without affecting the formation of uracil (Lion, 1968). Inasmuch as -SH compounds exist in cells, their reaction with DNA could explain the excess of uracils over single-strand breaks (in alkali).

DISCUSSION

Fig. 1 implies that most lesions that appear as alkali-labile bonds after ultraviolet irradiation of bromouracil-DNA in Tris buffer are single-strand breaks for irradiation in cells. This could be the action of some enzyme which converts alkali-labile bonds to breaks even at ice temperature. Another possibility is that the photochemical event initiates a series of reactions, and that the chemical environment can influence the

TABLE I

LESIONS PRODUCED BY 313 NM RADIATION IN BROMOURACIL-DNA IN *E. COLI* CELLS

Lesion	Approximate cross section* at 313 nm, $m^2/J \times 10^7$	Number relative to the single-strand break measured in alkali
Single-strand break (alkali)	4.5	(1.0)
True single-strand break (neutral)	3.4	0.7
Alkali-labile bond	1.2	0.3
Uracil production	7.6	1.5
Double-strand break	0.05	0.01

*The cross section is expressed per bromouracil base. The values are approximate because the adsorption of bromouracil varies rapidly with wavelength in the vicinity of 313 nm, so that the measurements are sensitive to its effective wavelength and hence to the way in which the "313 nm" radiation is prepared.

pathways and therefore the ratio of alkali-labile bonds to actual breaks that will occur. The fact that -SH compounds can greatly suppress the appearance of both lesions suggests that this latter possibility is not unreasonable.

The data in this paper and in the previous one (Krasin and Hutchinson, 1978) make possible the construction of Table I, lesions produced by 313 nm radiation in *E. coli* DNA containing bromouracil.

We thank Ms. Judith Stein for excellent technical assistance in experiments with λ DNA. We also thank Dr. Kenneth Beattie for calling attention to particular experimental uncertainties.

This research was supported by Department of Energy Research Contract EY-76-S-02-3571.

Received for publication 22 May 1978 and in revised form 27 July 1978.

REFERENCES

GEIDUSCHEK, E. P. 1962. On the factors controlling the reversibility of DNA denaturation. *J. Mol. Biol.* **4**:467.

HEWITT, R. R., and K. MARBURGER. 1975. The photolability of DNA containing 5-bromouracil. *Photochem. Photobiol.* **21**:413.

HUTCHINSON, F. 1973. The lesions produced by ultraviolet light in DNA containing bromouracil. *Q. Rev. Biophys.* **6**:201.

HUTCHINSON, F., and W. KÖHNLEIN. 1978. The photochemistry of 5-bromouracil and 5-iodouracil in DNA. *Prog. Mol. Subcell. Biol.* In press.

KRASIN, F., and F. HUTCHINSON. 1978. Double-strand breaks from single photochemical events in DNA containing 5-bromouracil. *Biophys. J.* **24**:645.

LEVIN, D., and F. HUTCHINSON. 1973. Relation between single-strand DNA mass and sedimentation distance in alkaline sucrose gradients. *J. Mol. Biol.* **75**:495.

LION, M. 1968. Search for a mechanism for the increased sensitivity of bromouracil-substituted DNA to ultraviolet radiation. *Biochim. Biophys. Acta.* **155**:505.

ROSENBERG, A. H., and F. W. STUDIER. 1969. Intrinsic viscosity of native and single-stranded T7 DNA and its relationship to sedimentation coefficient. *Biopolymers.* **7**:765.

ROSS, P., and P. HOWARD-FLANDERS. 1977. Initiation of *recA*$^+$-dependent recombination in *E. coli* (λ). I. Undamaged covalent circular λ DNA molecules in *uvrA*$^+$ *recA*$^+$ lysogenic host cells are cut following superinfection with psoralen-damaged λ phages. *J. Mol. Biol.* **117**:137.

RYDBERG, B. 1977. Discrimination between bromouracil and thymine for uptake into DNA in *drm*$^-$ and *dra*$^-$ mutants of *E. coli* K12. *Biochim. Biophys. Acta.* **476**:32.

STUDIER, F. W. 1965. Sedimentation studies of the size and shape of DNA. *J. Mol. Biol.* **11**:373.