DAMAGE AND REPAIR OF DNA IN 5-BROMODEOXYURIDINE-LABELED CHINESE HAMSTER CELLS EXPOSED TO FLUORESCENT LIGHT

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ABSTRACT Illumination of Chinese hamster cells with fluorescent light after 5-bromodeoxyuridine incorporation leads to extensive single-strand breakage in the DNA of the exposed cells. The rate of production of single-strand breaks is dependent on the extent to which thymine is replaced by 5-bromouracil. At least some of the breaks observed with alkaline gradients are probably produced in vivo and are probably not contingent upon alkaline hydrolysis since breakage can be demonstrated with neutral gradients also. Cells are able to rejoin most of the single-strand breaks within 60 min; however, damage to the DNA-containing material (the "complex") initially released from cells is repaired more slowly. Cysteamine protects against single-strand breakage with a dose-modifying factor of 2.8. A comparison is made between the production of single-strand breaks by fluorescent light and X-rays, and the significance of such breaks relative to cell survival is discussed.

INTRODUCTION

When thymine is replaced by 5-bromouracil (BU) in the DNA of viruses (Stahl et al., 1961) and mammalian cells (Puck and Kao, 1967), they become sensitive to fluorescent light (FL). 5-Bromodeoxyuridine (BUdR) incorporation also sensitizes cells to ionizing radiation as well as to ultraviolet (UV) light (for review see Elkind and Whitmore, 1967). The mechanisms of BUdR sensitization to X-irradiation are not completely understood (Shipley et al., 1971). Sensitization to UV irradiation may be the result of photochemically induced single-strand breaks which are produced in BU-substituted DNA of viruses (Lion, 1970), bacteria (Hutchinson and Hales, 1970), and mammalian cells (Smets and Cornelis, 1971). Sensitization of cells containing BU-substituted DNA to FL differs qualitatively from UV sensitization. On normal cells FL has no effect (Puck and Kao, 1967) whereas UV kills normal as well as BUdR-substituted cells. The only photoproduct produced when BU-substituted DNA is exposed to FL is uracil (Wacker et al., 1962). Since uracil

codes like thymine, it is not expected to be lethal and, therefore, the question of what is the lethal lesion remains without an answer. In UV-irradiated, BU-substituted T4 phage, damage to the deoxyribose moiety of DNA was observed and it was suggested that the production of uracil is accompanied by hydrogen abstraction from the deoxyribose (Hotz and Rueschl, 1967).

In the present work we studied the response of cultured Chinese hamster cells to FL after BUdR incorporation, relating damage to the DNA and its repair to cell survival. Using the alkaline sucrose gradient technique first introduced by McGrath and Williams (1966) and as modified by Elkind and Kamper (1970) we find that single-strand breaks are produced very efficiently and their production is closely correlated with the lethal effect.

MATERIALS AND METHODS

Cell Line

Chinese hamster fibroblasts, subline *V79-753B-3M*, were grown attached to glass or plastic in EM-15t (Shipley et al., 1971) and doubled in number in 8–9 hr.

Fluorescent Light Illumination

Before illumination, the medium in dishes was sucked out and Dulbecco's buffer added back. Illumination of the cells was with two tubular fluorescent lights (F15T8-D, GTE Sylvania, Inc., Mountain View, Calif.) held in a conventional desk top reflector at a distance of 2.5 cm, and was at ~3°C. The energy flux of the incident light under these conditions was 780 ergs mm^{-2} sec^{-1} as determined by a thermopile (Eppley Laboratory, Inc., Newport, R.I.) which was calibrated by a U.S. Bureau of Standards standard lamp.

Ultracentrifugation

To label DNA, cells were grown for 20 hr at 37°C in the presence of 1.0 μCi/ml thymidine-^{3}H, 6.0 Ci/mM (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.) together with 1×10^{-6} M BUdR. When 1×10^{-5} M BUdR was used, the thymidine-^{3}H activity was increased to 3.0 μCi/ml in order to obtain about the same amount of incorporation. The total thymidine concentration in the medium was kept at 5×10^{-7} M by adjusting the concentration of unlabeled thymidine appropriately. After illumination, an aliquot of a cell suspension obtained by trypsinization in the cold was pipetted onto 5–20% alkaline or neutral sucrose gradients. The labeled contents from cells were centrifuged in a Spinco L2-50HV ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) using an SW-50.1 rotor. 10-drop fractions were collected on glass fiber filters and the radioactivity counted as previously described (Elkind and Kamper, 1970).

Calculation of Molecular Weights and Number of Breaks

To determine a number average molecular weight (M_n), first, the molecular weight of each fraction in the gradient was calculated according to Studier's relationships (Studier, 1965). T4 phage DNA was used as a marker and the molecular weight of the duplex molecules was assumed to be 1.34×10^8 daltons (Rubenstein et al., 1961). The weight average molecular

weight (M_w) was then calculated from the equation

$$M_w = \sum f_i \cdot M_i, \tag{1}$$

where f_i is the fraction of radioactivity in the ith fraction and M_i is the molecular weight of the DNA in that fraction. M_n was taken as 0.6 M_w (Veatch and Okada, 1969). The number of breaks (N) produced per DNA molecule was calculated from the equation

$$N = \frac{M_n}{M_n^*} - 1 \tag{2}$$

where M_n is the number average molecular weight of the DNA from unirradiated cells and M_n^* is the number average molecular weight after irradiation.

RESULTS

Production of Single-Strand Breaks

In unsubstituted cells, 4 hr of lysis at 25°C in alkali before ultracentrifugation generally is required in order to release single-stranded DNA, "main peak" DNA, from a heterogeneous material to which we have referred as the complex (Elkind and Kamper, 1970). When BU replaces thymine in the cells' DNA, the release of single-stranded DNA requires only 60 min at 25°C (Shipley and Elkind, 1971). To study the complex, lysis was carried out at ~3°C for 2 hr and centrifugation was at 5000 rpm for 16.25 hr at 0°C (Elkind, 1971). Under these conditions at most only a small amount of single-stranded DNA is released from the complex. The complex is found near the bottom of the tube while the main peak is near the top (Elkind, 1971).

Qualitatively, increasing the dose of FL to BUdR-containing cells has the same effect as increasing X-ray doses to normal or BUdR-containing cells (Elkind and Kamper, 1970; Elkind, 1971; Shipley and Elkind, 1971). Small doses of FL cause the release of the main peak DNA from the complex; higher doses cause single-strand breakage as demonstrated in Fig. 1. In Fig. 1 *a*, the lysis period (90 min) was long enough to resolve the complex without FL; a 15 min FL exposure (7% survival) shifts the mode of the main peak from its usual location at about fraction 17 to fraction 8–9. In panel *b*, the shorter lysis period (30 min) is not long enough by itself to resolve the complex; some main peak DNA is evident at fraction 16 while appreciable complex remains in this case at fraction 18 judging from the characteristic sharp, single-point peak at this fraction. A 30 min exposure to FL (0.5% survival), however, produces a larger reduction in sedimentation than 15 min, which is clear even though a shorter lysis period was used. The remainder of the data in Fig. 1 will be discussed presently.

Fig. 2 shows the dependence of the number of single-strand breaks produced in the DNA of BUdR-containing cells on FL exposure under different conditions.

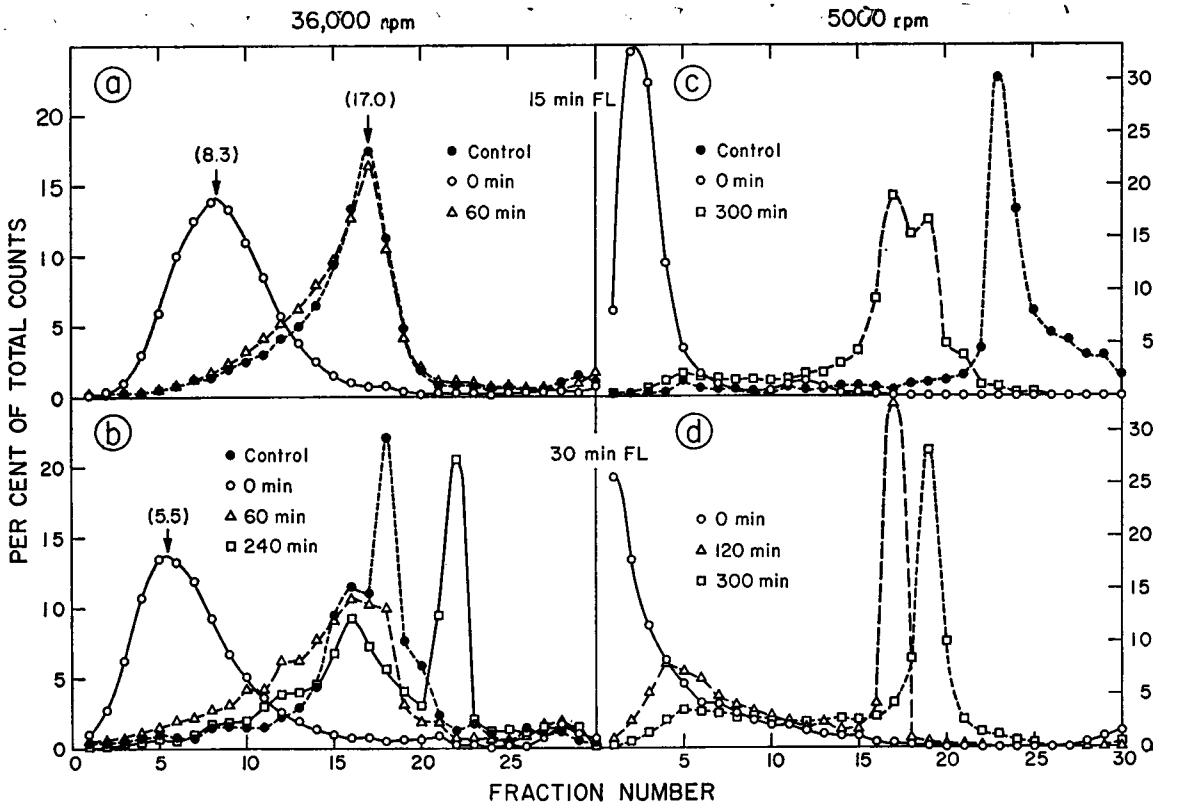


FIGURE 1 Sedimentation in alkaline sucrose gradients of DNA from Chinese hamster cells grown for 20 hr with 1×10^{-6} M BUdR and 1.0 μCi/ml thymidine-^{3}H. Cells (~5000) were lysed (90 min at 25°C for *a*, 30 min at 25°C for *b*, 120 min at ~3°C for *c* and *d*) in a 0.25 ml top layer (0.45 N NaOH, 0.5 M NaCl, 0.01 M Na_2-ethylenediaminetetraacetate (EDTA), pH 13.2) on top of 5–20% alkaline sucrose gradients (0.3 N NaOH, 0.7 M NaCl, and 0.003 M Na_2EDTA). Sedimentation: *a* and *b*, 36,000 rpm, ~12°C or 60 min, *c* and *d*, 5000 rpm, ~0°C for 16.25 hr. (*a*) Control, unilluminated cells, ●; cells exposed for 15 min to FL, ○; and cells incubated at 37°C for 60 min after 15 min FL, △; (*b*) Control, unilluminated cells, ●; cells exposed for 30 min to FL, ○; cells incubated at 37°C for 60 min after exposure, △; and cells incubated at 37°C for 240 min after exposure, □. (*c*) Control, unilluminated cells, ●; cells exposed for 15 min to FL, ○; and cells incubated at 37°C for 300 min after 15 min FL, △. (*d*) Cells were exposed for 30 min to FL. Without added incubation at 37°C, ○; with 120 min incubation at 37°C, △; with 300 min at 37°C, □. Recovery of radioactivity was greater than 80% in each gradient. The numbers in parentheses locate the modes. Direction of sedimentation was from left to right.

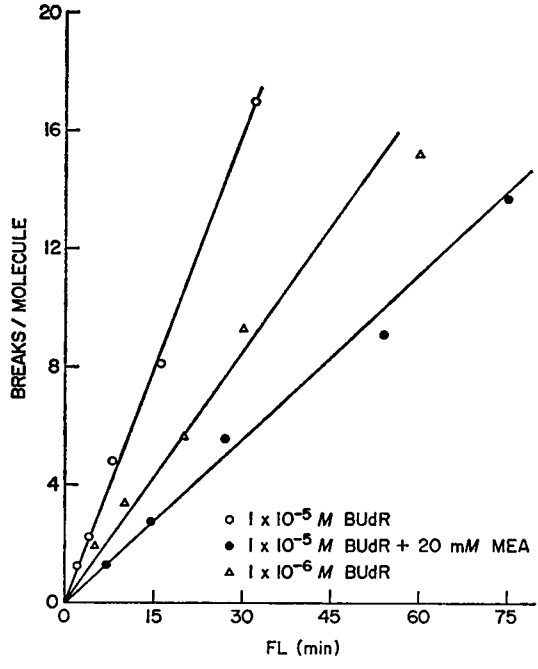


FIGURE 2 Number of breaks produced per single-stranded DNA (2×10^8 daltons) as a function of exposure time to FL. ○, cells grown for 20 hr with 1×10^{-5} M BUdR and 3.0 μCi/ml thymidine-^{3}H before exposure; △, cells grown for 20 hr with 1×10^{-6} M BUdR and 1.0 μCi/ml thymidine-^{3}H; ●, cells grown with 1×10^{-5} M BUdR and 3.0 μCi/ml thymidine-^{3}H for 20 hr and illuminated in the presence of 20 mM cysteamine·HCl (MEA). Illuminated cells were lysed on alkaline sucrose gradients, sedimented at 36,000 rpm, and the number of breaks determined as described in Materials and Methods.

It is evident that in the exposure range used, a linear relationship exists between FL exposure and the number of single-strand breaks produced. Furthermore, the rate of break production increases with increasing BUdR concentration in the medium since the slope of the line with 1×10^{-5} M BUdR is 1.9 times steeper than that with 1×10^{-6} M BUdR. Addition of cysteamine·HCl (mercaptoethylamine [MEA]) at a concentration of 20 mM present only during illumination, protected against the production of breaks by a factor of 2.8 as estimated from the ratio of the slopes (Fig. 2).

Repair of Single-Strand Breaks

Repair of single-strand breaks is demonstrable with the alkaline sucrose gradient technique by a return of the sedimentation profile of the DNA from irradiated cells to a profile more like that obtained from unirradiated cells. To effect repair after irradiation, cells are incubated before they are lysed. According to this criterion, essentially all the damage which results in single-strand breaks, produced by a 15 min exposure to FL (7% survival) in BUdR-substituted cells, is repaired if

the cells are incubated for 60 min in medium at 37°C, as shown in Fig. 1 *a*. When the length of the exposure to FL is increased to 30 min, a complete repair of the breaks produced requires a longer incubation period. In panel *b* of Fig. 1, after 60 min of incubation for repair a bulge remains to the left of the main peak. After 240 min, the bulge is further reduced and an appreciable proportion of the DNA sediments once again as a complex (sharp peak at fraction 22).

In Fig. 3 the kinetics of the repair of damage resulting in single-strand breaks after 30 min FL are described as the reduction in the percentage of breaks remaining as a function of incubation time at 37°C before lysis. A plot for the repair of X-ray–induced breaks is included for comparison; an exposure of 20 kR produced about the same number of breaks as 30 min FL. It can be seen that the rate of repair is similar in the two cases aside from the brief delay in the onset of repair in the cells that had been treated with X-rays. The upward displacement of the X-ray curve from the FL curve suggests that if all of the X-ray breaks are ultimately repaired after 20 kR, it takes appreciably longer for this than after FL. Ormerod and Stevens (1971) find that in murine lymphoma cells X-irradiated with doses exceeding 5 kR, only ~75% of the breaks are rejoined in ~60 min. The rest

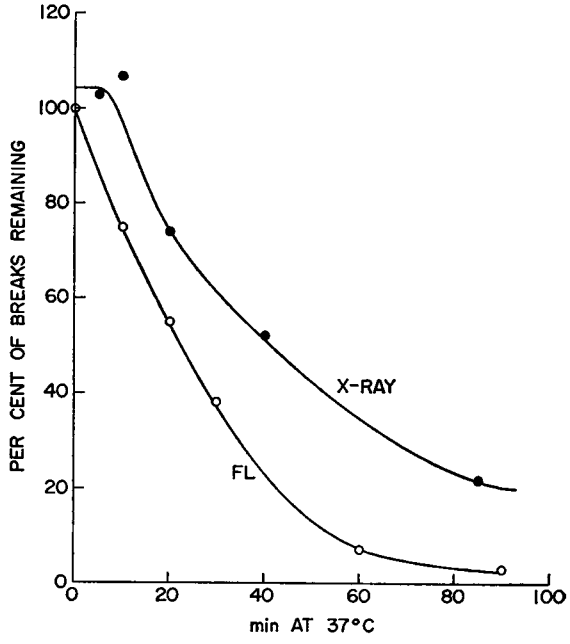


FIGURE 3 The rate of repair of single-strand breaks. Cells were grown for 20 hr with 1×10^{-6} M BUdR and 3 μCi/ml thymidine-^{3}H, illuminated for 30 min with FL (○), or exposed to 20 kR X-rays (250 kvp, 658 R/min) at ice temperature (●), and subsequently incubated for the indicated length of time at 37°C and the number of breaks determined after sedimentation in alkaline sucrose gradients for 17.1 hr at 11,000 rpm, ~3°C.

of the breaks remain unrepaired for over 2 hr; however, when Chinese hamster cells were X-irradiated with doses below 5 kR the breaks were rejoined completely within 20 min (Elkind and Kamper, 1970).

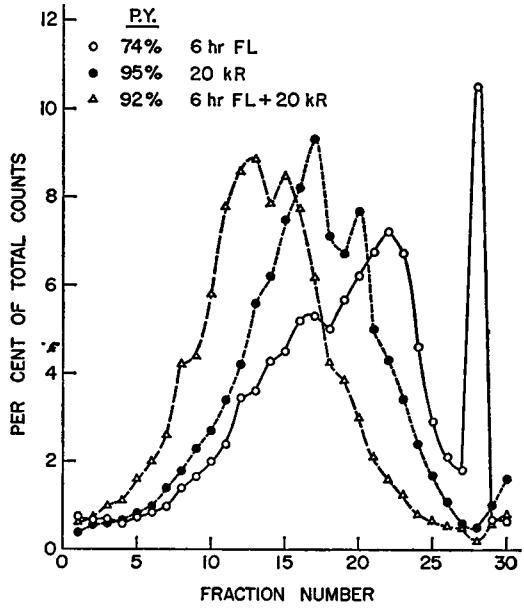
Initial repair of single-strand breaks caused by FL does not require the presence of complete growth medium. Repair of single breaks, apparently supported by endogenous reserves, can take place in Dulbecco's buffer at 37°C for periods up to about 60 min. Longer periods of incubation lead to degradation of main peak DNA, however. As in the instance of X-ray–induced breaks in normal or BUdR-containing cells, repair is suppressed at 0°C.

Repair of the Complex

Studies of repair of damage to the complex are facilitated by using slow speed sedimentation since as has been shown (Elkind, 1971), the complex sediments much more rapidly than main peak DNA at slow speed permitting thereby a clearer separation of these peaks. After X-ray doses in the high survival range (100–700 rads, 90–5% survival) Chinese hamster cells rapidly repair damage to the complex (Elkind and Kamper, 1970). In addition to the reappearance of the complex with repair that is evident in Fig. 1 *b* (36,000 rpm), in Figs. 1 *c* and *d* (5000 rpm) incubation up to 5 hr at 37°C after FL exposure results in DNA sedimenting in the lower third of the tube which otherwise would have sedimented near the top. Thus, although at a slower rate compared with the situation after X-irradiation, after FL (as well as after X-irradiation) Chinese hamster cells can reverse the resolution of main peak DNA from the complex effected by FL exposure.

The Nature of the Breaks Produced by FL

X-ray–induced single-strand breaks observed on alkaline sucrose gradients very likely exist before cell lysis in view of the large energy losses per absorption event. Hutchinson and Hales (1970) suggest that UV-induced single-strand breaks in BU-substituted DNA are formed *in situ* and are not the result of the alkaline conditions, since a similar yield of breaks was observed at neutral pH after formamide denaturation of the DNA. If after FL exposure the breaks are not simply induced by the alkaline conditions, they should be observable on neutral sucrose gradients as double-strand breaks after large enough FL exposure for single breaks in opposite strands to overlap. This was shown to happen in the case of UV-induced breaks in BUdR-substituted human kidney cells (Smets and Cornelis, 1971). Fig. 4 shows that this indeed happens in the case of FL-induced breaks. Under the conditions of sedimentation and lysis that were used, DNA from unirradiated cells sediments to the bottom of the tube. Exposure to 6 hr of FL results in a sedimentation profile in the lower third of the tube. If the cells are exposed to FL after X-irradiation, there is a further shift in the sedimentation profile toward the upper portion of the tube. This



RE 4 Sedimentation in neutral sucrose gradients of DNA from Chinese hamster cells n for 20 hr with 1×10^{-6} M BUdR and 6.0 μCi/ml BUdR-^{3}H (9.3 Ci/mM). Cells)00) were lysed (6 hr at ~25°C) in a 0.25 ml top layer (0.02 M Tris-Cl, 0.01 M Na_2EDTA, NaCl, 0.5% lauryldimethylamine oxide, 0.2% deoxycholate) on top of 5–20% neutral ıse gradients (2 M NaCl, 0.01 M Na_2EDTA, 0.015 M Tris-Cl, pH 8.6). Sedimentation ıt 8000 rpm for 16 hr at 20°C. Direction of sedimentation was from left to right. Cells sed for 6 hr to FL, O; cells irradiated with 20 kR X-rays (250 kvp, 658 R/min), ●; adiated cells (20 kR) exposed for 6 hr to FL, △. P.Y., per cent yield.

tation behavior is the result of double-strand breakage which is qualitamilar to that resulting from X-irradiation alone. Quantitatively, however, FL exposure after 20 kR reduces M_n from 6.6×10^8 to 3.3×10^8, corre- g to an average of only one double-strand break. An extrapolation of the n Fig. 2 suggests that a similar FL exposure would result in ~200 single- reaks/2×10^8 daltons, which is equivalent to ~660 single-breaks/1 double- ›reak in a molecule of 6.6×10^8 daltons. (The number of single-strand nduced by the added 20 kR dose is negligible, ~30.) This is to be compared ratio of about 1 double-strand break for each 7–10 single-strand breaks d by ionizing radiation (Freifelder, 1966; Corry and Cole, 1968; Veatch ıda, 1969). The 70–100-fold higher efficiency of ionizing radiation in pro- louble-strand breaks compared with FL is to be expected if, for X-rays, mainly the result of single absorption events and if those double breaks ; from FL exposure are, as is probable, the result of double absorption

Single-Strand Breaks Induced by FL vs. X-Ray

It is of interest to compare the extent of DNA damage induced by FL in BUdR-substituted cells with X-ray damage in the DNA of normal and BUdR-substituted cells to gain some additional insight into the importance of these types of lesions in the killing of cells. In Fig. 5 the break production by X-rays in normal and BUdR-substituted cells is compared. The efficiency of break production, calculated from the slopes of the curves, is 66 and 53 ev/break respectively. The apparently higher break efficiency in BUdR cells may be due in some small part to the greater sensitivity of BUdR-substituted DNA to alkaline conditions (Shipley and Elkind, 1971). Since care was taken to minimize this effect by lysing cells in the cold (~3°C), we believe that the data show a true sensitization by BUdR. Even if this ~20% enhancement of break formation is entirely attributed to sensitization by BUdR, however, it still would not account for the appreciable enhancement of cell killing which would result from the concentration of BUdR used (Shipley et al., 1971).

In Fig. 6, a comparison is made relative to cell killing between X-rays and FL-induced single-strand breaks by plotting the number of breaks against dose in units of the particular D_0 dose that is applicable. The points for the two BUdR concentrations in the case of FL fall on the same line because the ratio of the survival curve slopes equals the ratio of the corresponding break-induction curve slopes (Ben-Hur and Elkind, 1972). The two curves for X-rays differ mainly because of the reduction in D_0 which results from BUdR incorporation at this con-

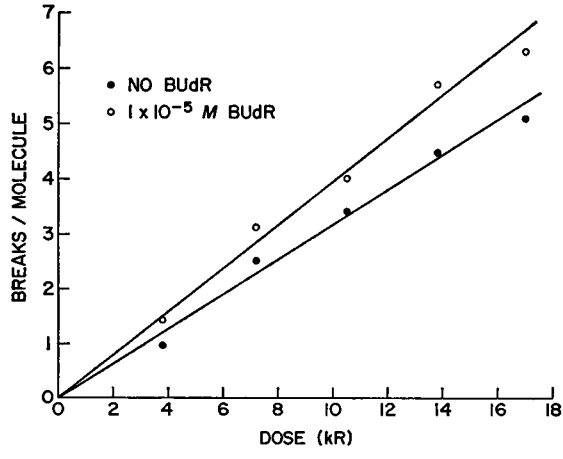


FIGURE 5 Number of breaks produced per single-stranded DNA molecule (2×10^8 daltons) as a function of X-ray dose. Cells were grown for 20 hr with 1×10^{-5} M BUdR and labeled with 9.0 μCi/ml thymidine-^{3}H (17.9 Ci/mM), ○; cells grown with 0.3 μCi/ml thymidine-^{14}C (37 mCi/mM), ●. The two cell populations were pooled, X-irradiated with 250 kvp at a dose rate of 656 R/min in ice, lysed on top of alkaline sucrose gradients for 5 hr at ~3°C, and centrifuged at 11,000 rpm for 17.1 hr at ~3°C. The number of breaks was determined as described in Materials and Methods.

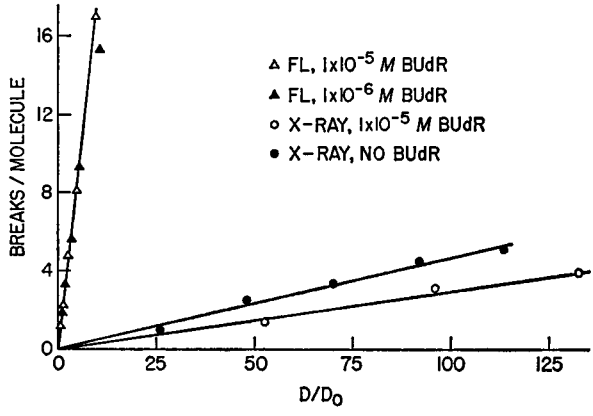


FIGURE 6 Number of breaks produced per single-stranded DNA (2×10^8 daltons) vs. dose in units of the D_0 dose. The FL data are the same as in Fig. 2, and the X-ray data are those of Fig. 5.

centration; the efficiency with which the breaks are produced in BUdR-containing cells is only slightly increased (Fig. 5). From Fig. 6 we see, therefore, that FL is much more efficient in producing single breaks in BUdR-substituted cells than is X-irradiation even in normal cells. FL is ~40 and ~60 times more efficient on a cell killing basis than are X-rays relative to normal and BUdR-substituted cells respectively.

DISCUSSION

Exposure of BUdR-substituted cells to FL leads to the resolution of the complex and the production of single-strand breaks in the DNA as demonstrated by the alkaline sucrose gradient technique. The number of breaks produced varies linearly with exposure and depends on the extent of BUdR substitution (for BUdR substitution see Ben-Hur and Elkind, 1972). The relative increase in the rate of break production, however, is smaller than the relative increase in the percentage of BUdR substitution; a similar situation has already been noted in regard to cell killing (Ben-Hur and Elkind, 1972). While the reasons for the lack of proportionality were not established, it is possible that there are regions of the genome where BUdR is photolyzed less readily, where photolysis has a less pronounced effect biologically, or both. Nonuniformity of chromosome breakage in Chinese hamster cells treated with BUdR has been shown by Hsu and Somers (1961).

Another factor affecting the rate of break production is the presence of cysteamine during FL exposure. The dose-modifying factor for cell killing and break production when 20 mM cysteamine is present during FL exposure is 2.1 (Ben-Hur and Elkind, 1972) and 2.8 respectively. A probable mechanism for the protection against the production of breaks by cysteamine is the one proposed for the protection against deoxyribose damage in UV-irradiated, BUdR-substituted T4 bacteriophage (Hotz

and Rueschl, 1967), namely the donation of a hydrogen atom by cysteamine to debrominated BU, thus producing uracil without concomitant hydrogen abstraction from the proximal deoxyribose moiety.

Using neutral sucrose gradients, double-strand breaks were observed after prolonged exposures to FL. The ratio of single to double-strand breaks was estimated to be ~700 after a 6 hr exposure. Since FL-induced double-strand breaks are probably the result of double absorption events, their production should be proportional to the square of the dose as was found for UV-irradiated BUdR-substituted human kidney cells (Smets and Cornelis, 1971). If they are the lesions responsible for cell death, the survival curve should be of the two-target, single-hit type or at least the damage accumulation type. As shown elsewhere, however (Ben-Hur and Elkind, 1972), the survival curves obtained are exponential or single-target, single-hit. Thus, although there is more than one double-strand break per cell per D_0, or lethal hit, this does not seem to be the cause of cell death.

As in the case of X-ray–induced single-strand breaks, FL-induced breaks are rejoined efficiently and at a similar rate. With regard to repair of the complex, after a survival equivalent dose the complex is repaired more slowly after FL exposure than after X-ray exposure. This point by itself suggests that the integrity of the complex may not be a critical determinant relative to *both* BUdR-FL killing and X-ray cell killing of normal or BUdR-containing cells; however, since repair of single breaks always precedes repair of the complex (Fig. 1 and Elkind and Kamper, 1970), the apparent slower rate of repair of the complex after FL could reflect the disproportionately greater number of single breaks produced by FL compared with X-rays. Alternatively, if integrity of the complex is important for cell survival, the suggestion follows that cell death after X-irradiation results from a lesion differing from that producing cell death after FL. Since BUdR sensitizes cells to FL, it is highly likely that lethality results from damage to DNA in view of the specific incorporation of BUdR into DNA. In view of this, the considerably lesser degree of sensitization produced by BUdR relative to X-irradiation further supports the idea that the X-ray lethal lesion differs from that due to FL, and therefore, in the case of X-rays, lethal damage may not be simply single breaks in DNA.

Fluorescent light induces ~1.5 single-strand breaks in 2×10^8 daltons per D_0 dose or $\sim 5 \times 10^4$ breaks/cell (determined from Fig. 6). These numbers are strikingly greater than those for X-rays (~40 to ~60 times) and as noted this fact alone makes single-strand breaks a good candidate for the cause of cell death after FL exposure. If this is the case, then repair of damage which results in single-strand breaks as the term has been used here would be indicative of lesion fixation, rather than lesion elimination, since a return to normal-appearing sedimentation ability does not mean a complete return to the undamaged state. Other evidences in support of a single-strand break killing mode are the similar degrees of added sensitization for cell killing and for single break production when the cells are grown with 1×10^{-5} M BUdR compared with 1×10^{-6} M BUdR (1.7 and 1.9 respectively) and

the similar degrees of protection when cysteamine is present during illumination (2.1 and 2.8 respectively).

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