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On the Mechanism of Radiosensitization by 5-Bromouracil Studies on ^{60}Co-gamma Irradiated Phage ϕX-174 and its Single-stranded Infectious DNA

UV-Sensitization of Phage ϕX-174 by 5-Bromouracil

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Summary. Experimental evidence for the sensitization of broth-suspended phage ΦX-174 and its single-stranded infectious DNA by incorporation of 5-bromouracil against ionizing radiation is given. No influence of phage protein or molecular DNA-structure on the amount of BU-damage has been observed. The absence of desensitization by radical-scavenging compounds like cystamine in single-stranded DNA is discussed. It is deduced from the experimental data that host-cell and/or phage depending reactivation processes are involved in sensitization and desensitization of BU-DNA.

Introduction

It has already been shown that particles of phage ΦX-174 containing BU-DNA are more sensitive to UV-light of 2,800 Å by a factor of 1.5 compared with normal phage (DENHARDT and SINSHEIMER, 1965b). The same observation holds for infectious BU-DNA isolated from this phage which is sensitized by a factor of 5.5 when irradiated at a wavelength of 3,130 Å (RÜST and SINSHEIMER, 1967).

However, to our knowledge no study of *single*-stranded BU-DNA using ionizing radiation is reported in the literature. Since it was shown that with ionizing radiation BU acts as a pronounced sensitizer in double-stranded phage T4 (STAHL et al., 1961) and T1 (HOTZ, 1963) it was of special interest to test a single-stranded phage in this respect. From a comparison of experiments with isolated single-stranded BU-DNA of phage ΦX-174 with the radiobiological effect of BU on whole phage particles further elucidation of the radiosensitizing mechanism of BU can be expected.

Special attention has been given to the desensitization phenomenon of compounds of the cysteine-cysteamine group, when present during irradiation of phage with UV as well as with ionizing radiation (HOTZ, 1963; HOTZ and ZIMMER, 1963). A first indication as to the nature of the mechanism of BU-sensitization came from studies using the electron-spin-resonance method (MÜLLER et al., 1963). It was shown that formation of one free radical induced by ionizing radiation requires 1,200 eV in thymine but only 150 eV in BU. Also, in UV-irradiated BU-DNA radicals have been observed which were attributed to the presence of BU (KOEHNLEIN and HUTCHINSON, 1966). Furthermore, there is evidence that uracil is the chief product of BU after inactivation of BU-substituted DNA with UV and ionizing radiation (WACKER, 1963; SMITH, 1964). This led to the conclusion that the main reactions of the radiosensitizing mechanism are dehalogenation of the BU and formation of uracil from a uracil radical by abstraction of hydrogen from a neighbouring deoxyribose group. Damage to the

deoxyribose could be proved by direct chemical analysis of the DNA of UV-irradiated BU-T4 phage (HOTZ and REUSCHL, 1967). It is proposed by these authors that the radical reaction can be interrupted by irradiation in the presence of a compound like cysteamine, which restitutes the H-atom to the uracil radical thus preventing the destruction of deoxyribose.

Material and Methods

Phage ΦX-174 wild-type, host *E. coli C* wild-type and *E. coli CR34/C416* were kindly supplied by Dr. R. L. SINSHEIMER (Pasadena, USA) in 1960 and 1965, respectively. *E. coli C/1*, a mutant resistant to phage T1 was selected from *C* wild-type and used for phage plating. *E. coli K12* was kindly supplied by Dr. P. STARLINGER (Cologne).

Phage DNA was prepared and assayed in an *E. coli K12* spheroplast system according to GUTHRIE and SINSHEIMER (1963). The technique of DENHARDT and SINSHEIMER (1965a) was followed to grow BU-ΦX-174. The last step in all phage purifications was a CsCl density-centrifugation. BU-ΦX was stored in borate buffer (BP) (sodium tetraborate solution saturated at 4°C) containing 20% nutrient-broth (NB). Plating of phage was done by our standard technique, spreading 0.1 ml of phage suspension with 0.1 ml of host cell overnight-culture on colored plates (HOTZ and MÜLLER, 1960).

The technique for irradiation with ^{60}Co-gamma radiation as well as the dosimetry were already described in detail (HOTZ and ZIMMER, 1963). Cystamine (2,2′-dithio-bis(ethylamine)-dihydrochloride) was a product of Calbiochem, Los Angeles. 10^9 bacteriophage particles per ml and about 0.1 μg infectious DNA per ml, respectively, were irradiated. As the criterion of radiation damage plaque-forming ability (PFA) of phage particles and of their infectious DNA was chosen.

Results

1. Sensitivity of BU-ΦX Phage to ^{60}Co-gamma Irradiation

Fig. 1 gives the data of experiments with BU-substituted ΦX-174 phage and with wild-type phage of normal radiosensitivity. Heavy BU-phage was purified by CsCl density-gradient centrifugation as described previously (HOTZ, 1968). Inactivation of phage particles was followed in 4% broth, a solution widely used to avoid almost all of the so-called indirect effect of ionizing radiation in aqueous solution. All phage used throughout the experiments reported in this communication are from the same batch analyzed by density-gradient centrifugation which means that the amount of thymine substituted by BU is equal in all samples. From these experiments it is evident that:

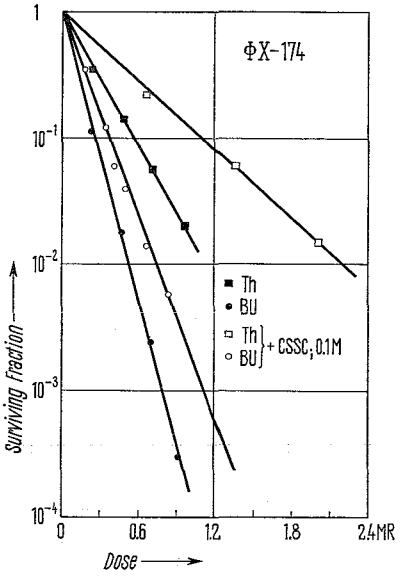

Fig. 1. Surviving fraction (*PFA*) of normal (*Th*) and BU-substituted (*BU*) phage ΦX-174 suspended in 4 per cent Difco-nutrient broth after aerobic irradiation with a ^{60}Cobalt gamma-source. The curves represent the survival in the presence and absence of M/10 cystamine dihydrochloride (*CSSC*) at pH 7.5

(1) The phage is strongly sensitized against ionizing radiation by incorporation of BU. The sensitization factor amounts to $F_s = 2$.

(2) Cystamine in normal ΦX-phage gives a protection factor of $F_p = 1.9$ against gamma radiation which is comparable to experiments with phage containing double-stranded DNA (Hotz, 1966).

(3) With BU-phage gamma-irradiated in the presence of cystamine, however, only a certain amount of sensitization seems to be suppressed and the protection factor is about 1.3.

The following reasons led us to choose the disulfide cystamine for the experiments with ^{60}Co-gamma radiation: Application of a concentration of M/10 cysteamine [optimal for eliminating the BU-effect in gamma-irradiated T1 (Hotz, 1963)] to ΦX-174 phages and their DNA turned out to be very toxic, whereas cystamine [a compound that is able to abolish the sensitizing effect in BU-T1 as well as the thiol (Uenzelmann, 1968)] was found to be much less toxic.

2. Sensitivity of Infectious BU-ΦX DNA to ^{60}Co-gamma Irradiation

Fig. 2 shows the results of experiments identical to those described in the foregoing chapter performed with infectious DNA isolated from phage ΦX-174 and irradiated as free molecules in suspension. We conclude that:

(1) BU-incorporation renders the free DNA even more sensitive to ionizing radiation compared with DNA irradiated inside the protein coat of the phage. The sensitizing factor is $F_s = 3.5$.

(2) Cystamine gives a protective effect in normal infectious DNA of $F_p = 3.5$, and in BU-DNA of $F_p = 6$.

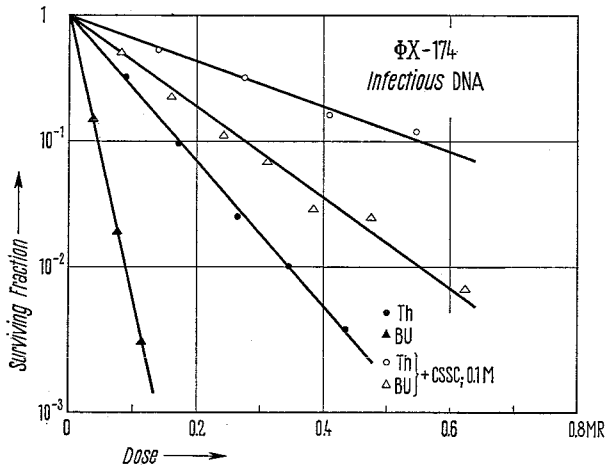

Fig. 2. Surviving fraction (*PFA*) of infectious DNA isolated from phage ΦX-174 before irradiation with a ^{60}Cobalt gamma-source. The irradiations are done in air on DNA suspended in 4 per cent Difco-nutrient broth. The survival of normal (*Th*) and BU-substituted (*BU*) DNA in the presence and absence of M/10 cystamine dihydrochloride (*CSSC*) at pH 7.5 is shown in corresponding curves

Fig. 3 summarizes and gives a comparison of the relative and absolute radiosensitivity measured in our experiments. From this it is evident that although a pronounced protective effect of cystamine is observed no reduction of sensitization due to the base analogue is found in BU-phage and just a small reduction in free BU-DNA.

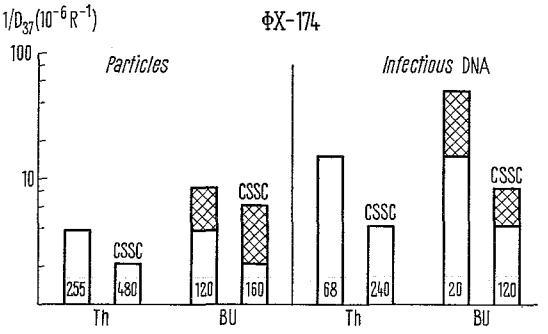

Fig. 3. Reciprocal of 37 per cent survival dose (D_{37}) of normal (*Th*) and BU-substituted (*BU*) ΦX-174 phage particles and their infectious DNA, respectively. The data are taken from the experiments shown in Fig. 1 and Fig. 2. *CSSC* indicates the presence of cystamine during irradiation and the numbers in the columns refer to the absolute D_{37} in krad. The marked parts of the columns represent the additional radiation damage due to BU-incorporation (sensitization)

Discussion

The results of our experiments with ^{60}Co-gamma radiation can be summarized as follows:

(i) The amount of additional BU-damage in phage ΦX-174 expressed in the sensitization factor (F_s) is comparable to that occurring in double-stranded phage.

(ii) The same observation holds for infectious ΦX-DNA irradiated in the isolated state.

(iii) Irradiation of broth suspended normal phage ΦX-174 or infectious DNA in the presence of cystamine results in radioprotection already known as hyperprotection.

(iv) Irradiation of BU-substituted phage ΦX-174 in the presence of cystamine does not result in reducing BU-sensitization.

(v) Irradiation of free BU-DNA in the presence of the radical scavenger only partially abolishes the sensitizing effect of BU.

Result (i) indicates that the molecular structur of DNA, i.e. single- or double-strandedness, does not influence the BU-effect. The same is concluded from result (ii) on free BU-DNA, a system which can be compared with BU-substituted transforming DNA (Opara-Kubinska et al., 1961). Result (ii) is also consistent with the assumption that the principal target of the BU-damage is the DNA and that the phage protein is not involved in the mechanism of sensitization by the base analogue. The most plausible explanation for the fact that the sensiziting effect of BU is higher in infectious DNA than in phage is that BU-incorporation leads to general fragility of the DNA molecule (Szybalski and Opara-Kubinska, 1964), which can be expected to give an increased BU-effect on a DNA molecule floating in suspension.

Discussion of the mechanism of hyperprotection in phage is not subject of this communication since this problem was already discussed in detail (Hotz and Zimmer, 1963; Howard-Flanders et al., 1963; Hotz, 1966). The same mechanism should hold for single-stranded DNA and it is obvious from our results (iii) that the radioprotective chemicals reduce lesions in the DNA strand which has been hit by an inactivating event and that the protective action on the phage protein is negligible at doses of biological interest compared with the action on DNA.

At the moment we can only speculate why desensizitation is not observed under all experimental conditions. Studies on UV-irradiated phage containing double-stranded DNA suggest that BU-damage modified by radical scavengers can be reactivated by the host cell (Hotz, 1963, 1964; Hotz and Reuschl, 1967). Furthermore, there is evidence that normal phage T1 damaged by ionizing radiation can partially be reactivated by the host cell (Sauerbier, 1964). Reactivation ability of the host for BU-damage which is modified when the phage is irradiated in the presence of a radical scavenger would be an explanation for desensitization observed in T1 (Hotz and Zimmer, 1963; Uenzelmann, 1968) and in T7 (Freifelder and Freifelder, 1966) when inactivated by ionizing radiation. If we assume that in single-stranded DNA of ΦX-174 the effect of compounds of the cysteine-cystamine group on the physico-chemical reaction of BU during irradiation is the same as in the double-stranded BU-DNA, and if we furthermore postulate that biological repair mechanisms are involved in desensitization (Hotz, 1968) no influence of a radical scavenger can be tested by a biological assay in single-stranded BU-DNA, e.g. in ΦX-174, since no reactivation of damaged DNA has been observed in this phage. Our results obtained with BU-ΦX do fit this assumption.

In the case of isolated ΦX-DNA the sector of damage due to the BU-effect is reduced from 70% in the absence down to 50% in the presence of cystamine. It may be noted, however, that the BU-effect is more pronounced in free ΦX-DNA than in corresponding phage particles. At the moment it cannot be excluded that this is due to reactive species, e.g. OH-radicals, arising from the irradiated suspension medium (Blok et al., 1962). If this assumption is correct we should expect an influence of a radical scavenger which would show up as partial desensitization. The radiochemical reactions of BU-DNA irradiated in the isolated state have been studied much less than those of phage particles and further results especially on infectious double-stranded BU-DNA should be awaited before we shall be able to understand completely the mechanism of radiosensitization by 5-bromouracil. At present we may attempt to improve existing models of DNA-inactivation as the one proposed recently by Szybalski (1966).

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UV-Sensitization of Phage ΦX-174 by 5-Bromouracil

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Summary. Experimental evidence for the sensitization of buffer-suspended phage ΦX-174 by incorporation of 5-bromouracil against UV-light of 2,537 Å is given. The significance of missing desensitization by radical-scavenging compounds like cysteamine in single-stranded DNA is discussed. It is concluded tentatively that host-cell and/or phage depending reactivation processes are involved in sensitization and desensitization of BU-DNA.

Introduction

Radiosensitization by incorporation of the base analogue 5-bromouracil (BU) into the DNA of different phage types has been studied by several investigators. However, almost all studies have been performed with objects containing *double*-stranded DNA. The present communication is concerned with the radiobiological effect of BU on phage ΦX-174 containing *single*-stranded DNA. Further elucidation of the radiosensitizing mechanism of BU can be expected from such experiments.

It has already been shown that phage ΦX-174 containing BU-DNA is more sensitive to UV-light (Kozinski and Szybalski, 1959; Denhardt and Sinsheimer, 1965b) compared with normal phage particles. Recently it was observed (Hotz and Reuschl, 1967) that UV-irradiation of phage BU-T4 is followed by damage to the deoxyribose of DNA, a lesion which was avoided by the presence of cysteamine (2-mercaptoethylamine) during irradiation. No damage to the deoxyribose of normal phage was detected. It was concluded tentatively that an undamaged sugar-phosphate backbone of DNA is essential for the action of enzymes responsible for repairing different radiation lesions in double-stranded BU-less as well as in BU-DNA (Hotz and Reuschl, 1967). According to the theory of excision and reunion forwarded by Setlow and Carrier (1964) as well as by Boyce and Howard-Flanders (1964) such a repair mechanism is restricted to double-stranded DNA. Therefore in BU-ΦX no repair is expected even if irradiation is performed in the presence of cysteamine, as will be shown in the present communication.

Material and Methods

Phage ΦX-174 wild-type, host *E. coli C* wild-type and *E. coli CR34/C416* were kindly supplied by Dr. R. L. Sinsheimer (Pasadena, USA). ΦX-174*h* is a host range mutant selected from the wild-type strain for the special purpose of CsCl density-gradient centrifugation as a density reference. *E. coli C/1*, a mutant resistant to phage T1 was selected from *C* wild-type and used through almost all experiments. *E. coli C/1/ΦX* was selected from *C/1*. This mutant gives plaques after infection with ΦX-174*h* but not with wild-type phage. Assay of phage was done on *C/1* or *C/1/ΦX* respectively. The technique of Denhardt and Sinsheimer (1965a) was followed to grow BU-ΦX-174. The technique for plating the phage and for irradiation with UV-light of 2,537 Å as well as the dosimetry were already described in detail (Hotz and Zimmer, 1963). 10^9 bacteriophage particles per ml in M/15 phosphate buffer were irradiated.

For density-gradient analysis of the BU-phage 0.01—0.1 ml samples of appropriate dilution together with ΦX host range mutant were mixed with 4 ml CsCl-borate solution (0.53 g CsCl/ml). The suspension was filled into lusteriod tubes, with a top-layer of paraffin oil, and centrifuged to equilibrium in a SW 50 rotor of a Spinco model L2-65 at 35,000 R.P.M. for 20 hours. At the end of the run the bottom of the tube was pierced and the emerging drops collected in borate buffer in fractions of 5 drops. The particle density corresponding to the peaks found are calculated by measuring the refractive index (n_{D20}) of the fractions from the bottom and from the top of the tubes by an Abbe-refractometer (Zeiss, Germany). The density gradient of the system was found to be linear and the mass density corresponding to the refractive index was determined by weight measurements of CsCl-borate buffer solutions.

Results

1. Density-gradient Centrifugation

In order to prove incorporation of BU and to get an estimate of the amount of BU-substitution a sample of BU-ΦX was centrifuged to equilibrium in a CsCl density-gradient in the presence of a density reference phage (ΦX*h*) (Fig. 1). From the distribution of phage throughout the gradient it can be concluded that the particle density is homogeneous in ΦX wild-type and ΦX host range mutant. However, in BU-ΦX the density is less homogeneous, a phenomenon already observed by others (Denhardt and Sinsheimer, 1965a; Rüst and Sinsheimer, 1967).

It was pointed out by these authors that BU-DNA isolated from BU-particles showing this heterogeneity, bands into a sharp peak, and therefore possesses homogeneous density. The degree of substitution of thymine by BU in our particles has not been directly determined. The density of our BU-phage, however, is comparable to the value reported by Denhardt and Sinsheimer (1965a). Our data together with others reported in the literature are listed in the table.

Table. *Densities of various ΦX-174 preparations*

Material	Experimental density (g/ml)	Authors
ΦX-174	1.4	Sinsheimer, 1959
	1.451	Kozinski and Sybalski, 1959
	1.400	own measurements
ΦX*h*	1.408	own measurements
γh-ΦX	1.43	Denhardt and Sinsheimer, 1965a
BU-ΦX	1.458	Denhardt and Sinsheimer, 1965a
(heavy peak)	1.476	Kozinski and Szybalski, 1959
	1.442	own measurements
BU-DNA	1.807	Rüst and Sinsheimer, 1967

2. UV-sensitivity of BU-ΦX Phage

Fig. 2 shows the results of some experiments with BU-substituted ΦX-174 phage and with wild-type phage of normal radiosensitivity for reference. For UV-irradiation phage purified by CsCl density gradient-centrifugation are diluted

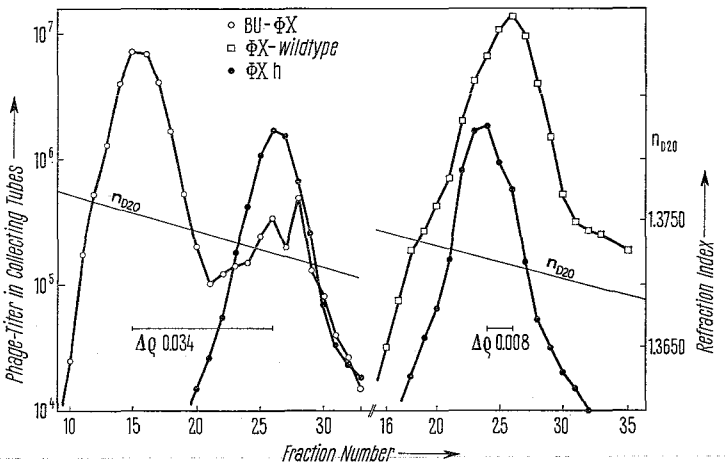

Fig. 1. Density distribution in a preparation of phage ΦX-174 substituted with 5-bromouracil and banded in CsCl-solution at 35,000 R.P.M. for 20 hrs. in a rotor SW 50 (Spinco). Distribution of the density-reference phage ΦX-174*h* and ΦX-174 wild-type is shown for comparison

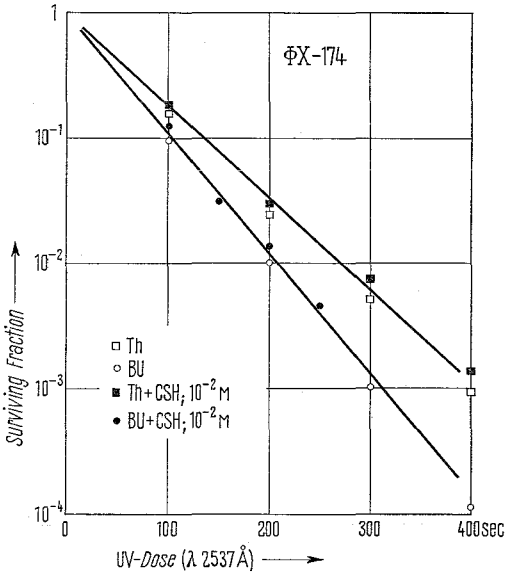

Fig. 2. Surviving fraction of the plaque-forming ability of purified normal (*Th*) and BU-substituted (*BU*) phage ΦX-174 after irradiation with UV-light in M/15 phosphate buffer (pH 7.2). The effect of M/100 cysteamine-base (*CSH*) present during irradiation of both phage is indicated by filled symbols

a hundredfold in M/15 phosphate buffer and the inactivation rate of the plaque-forming ability was measured in the absence and presence of a radical scavenger (cysteamine-base; Fluka, Switzerland; pH 7.2). It is evident from this diagram

that all curves are exponential and their slopes can be measured very accurately. The effect of BU-incorporation can be expressed in terms of sensitization (F_s) factors. The following aspects of this set of curves may be noted:

(i) Incorporation of BU into the single-stranded DNA sensitizes phage ΦX against UV-light of 2,537 Å by about 30% ($F_s = 1.3$).

(ii) The presence of M/100 cysteamine alters the radiosensitivity neither of normal nor of BU-substituted phage.

Discussion

In order to understand the first result (i) the wavelength dependence of UV-damage in BU-DNA and in normal single- or double-stranded DNA, respectively, must be considered. Damage to normal phage DNA was found to decrease much more rapidly than damage to BU-DNA when proceeding from the absorption maximum of normal DNA towards longer wavelengths of UV-light. Sensitivity in phage BU-ΦX irradiated at 2,360 Å is increased by a factor of 1.6, by 1.1 at 2,600 Å, but again by 1.4 at 2,700 Å and by 9 at 3,020 Å (Denhardt and Sinsheimer, 1965b), i.e. sensitization is smallest around the absorption maximum of normal DNA. Sensitization of double-stranded phage DNA by BU is largest under conditions where reactivation phenomena can be observed. However, even in the absence of such repair as host-cell reactivation (HCR) BU sensitizes UV-irradiated phage T1 to some extent (Hotz, 1964). The same conclusion can be drawn from the observed sensitization in phage BU-ΦX since no HCR can be observed with normal single-stranded ΦX-174 (Sauerbier, 1964). It is plausible to assume that the above mentioned facts reduce the BU-effect in UV-irradiated phage ΦX. We conclude that in addition to lesions in normal DNA two types of radiation damage are obviously due to BU-incorporation in phage DNA: The larger part of the BU-effect is due to blocking different host-cell and/or phage depending repair mechanisms (e.g. HCR, photoreactivation and *u*-gene reactivation) and can be detected only in phage-host systems possessing such mechanisms. The smaller part of BU-damage can be observed in phage-host systems lacking reactivation of UV-damage. For this effect an enhanced DNA breakdown of irradiated BU-DNA in the host and a restricted DNA-synthesis during replication by the host-cell could be responsible as would be analogous to the findings with UV-irradiated bacteria (Aoki et al., 1966).

Result (ii) is in contrast to double-stranded phages already tested, e.g. T1 (Hotz, 1963; Rupp and Prusoff, 1964), T4Bo^r (Hotz and Reuschl, 1967), and T2, T4, T4*x* (Hotz, unpublished results). In these phages cysteamine present during UV-irradiation abolishes the sensitizing effect of BU ("desensitization"). In the case of single-stranded DNA it may be expected that the effect of cysteamine on the physico-chemical reaction of BU (Hotz and Reuschl, 1967) during irradiation is the same as in the double-stranded molecule. If we assume, however, that biological repair mechanisms are involved in desensitization no influence of a radical scavenger can be assayed biologically in ΦX-174 since no reactivation has been observed as already mentioned above. The results obtained with BU-ΦX irradiated inside the host (Denhardt and Sinsheimer, 1965b) support this assumption since a pronounced BU-effect is observed under such experimental conditions. With double-stranded intracellular phage, however, no en-

hancement of radiosensitivity could be shown. It was assumed (HOTZ, 1963) that in a phage-host complex, natural sulfhydryl compounds present in the cytoplasm of the bacterium are acting in a way similar to the in-vitro reactions of cysteamine. Furthermore it is of interest that desensitization is also absent in transforming BU-DNA irradiated with UV-light (RUPPERT, personal communication). For this observation the fact might account that transforming activity is lost as the result of single-stranded lesions (SUMMERS and SZYBALSKI, 1967). This would parallel the above mentioned situation in single-stranded ΦX-DNA in which single-strand lesions are also lethal.

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