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ON THE MECHANISM OF RADIOSENSITIZATION BY 5-BROMOURACIL. THE OCCURRENCE OF DNA STRAND BREAKS IN U.V.-IRRADIATED PHAGE *T4* AS INFLUENCED BY CYSTEAMINE

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Abstract– U.V.-irradiation of phage *T4*Bo^r results in a decrease in sedimentation rate of BU-DNA which is attributed to single- and double-strand breaks. No breaks could be observed in unsubstituted DNA. Cysteamine present during u.v.-irradiation is able to prevent double-strand breaks but does not influence the production of single-strand breaks measured by alkaline sucrose gradient centrifugation. The biological importance and nature of DNA strand breaks due to BU-incorporation as well as the action of the protective agent on these breaks and on the biological activity are discussed.

INTRODUCTION

A FIRST indication as to the molecular events in u.v.-damaged DNA containing 5-bromouracil (BU) was given by Marmur *et al.*[1] who found evidence for a decreased sedimentation coefficient of *E. coli* DNA after irradiation *in vitro*. Compounds of the cysteine-cysteamine group, well known as efficient radical scavengers[2], can abolish sensitization of the plaque forming ability of BU-phage against u.v.-light and ionizing radiation[3] as well as u.v.-damage to the deoxyribose groups of BU-DNA[4] if irradiation is performed in the presence of these chemicals. This suggested radical reactions are involved in the radioinactivation of the biological activity of BU-phage. It was assumed by the authors[4] that damage to the deoxyribose would eventually lead to lesions of the sugar–phosphate backbone of the DNA. Kaplan[5] described an increase of double-strand scissions after X-irradiation of *E. coli* K-12 due to BU-incorporation. Lion[6] reported a preliminary study on single-strand breaks in the DNA of u.v.-inactivated phage BU-*T3* which could be partially prevented by irradiation in the presence of cysteamine. Inhibition of u.v.-induced chromosome breaks by cysteamine was observed in BU-substituted mammalian cells[7].

After having shown the effect of u.v.-light on the plaque-forming ability of BU-*T4* in the absence and presence of cysteamine[4], the purpose of this paper is to present evidence for the occurrence of strand breaks in u.v.-irradiated phage *T4* DNA if the phage is sensitized by BU-incorporation. Double-strand breaks are completely prevented if irradiation is performed in the presence of 0·01 *M* cysteamine. Single-strand breaks, however, assayed by a decreased sedimentation rate in an alkaline sucrose gradient were not subject to this effect.

MATERIALS AND METHODS

Strains. Coliphage *T4*Bo^r, an osmotic-shock resistant mutant of *T4* wild-type was originally supplied by Dr. W. Harm (Dallas) in 1961 and used throughout the experiments. The prototroph host *E. coli* B and the mutant *E. coli* CR34, *thr leu thy lac T1^r T5^r* were originally obtained from the Institut für Genetik in Köln.

Preparation of radioactive phage

5-bromouracil (2-^{14}C) *phage.* The technique of incorporation of radioactive BU (specific activity 14·5 μCi/mg; 20 μg/ml growth medium) into the phage followed a modified method of Stahl *et al.*[8]. The phage host was *E. coli* CR34. About 30 per cent of thymine was substituted by BU as was independently measured by determination of radioactivity and by measurement of the increase in density of the phage by CsCl-density-gradient centrifugation.

Thymine (2-^{14}C) *phage.* This technique of preparation followed the same line as mentioned above using ^{14}C-labelled thymine (spec. activity 17 μCi/mg; 20 μg/ml medium). Both compounds have been synthetized by Dr. Schweer (Institut für Strahlenchemie, Kernforschungszentrum Karlsruhe).

Phage were concentrated and purified by differential centrifugation. The last step of preparing the phage stock was a CsCl-density-gradient centrifugation followed by dialysis against adsorption-buffer of Hershey and Chase[9].

U.V.-irradiation. $T4Bo^r$ phage stock was diluted in phage buffer to about 3×10^{11} particles/ml. Under stirring 0·6 ml of the suspension contained in a small glass dish were exposed to a 6 W Hanau u.v.-lamp (mercury-vapor low-pressure, model NK 6/20) giving a dose-rate of 1·6 ergs/mm^2 . sec at a distance of 45 cm. In some experiments DNA already extracted from phage was irradiated at a concentration of 20 μg/ml NCE-buffer (0·179M NaCl, 0·02M Na-citrate, $10^{-3}M$ EDTA). When high u.v.-doses had to be delivered a specially designed low-pressure mercury-vapor lamp (Gräntzel, Karlsruhe; Vycor-glass) was used at a distance of 22 cm giving an intensity 54·5 times higher than our small u.v.-source. Both u.v.-sources emit about 95 per cent of their radiation at 2537 Å.

The u.v.-dose delivered to the phage particles and indicated in phage lethal hits (PLH) was measured by means of survival curves for each particular phage stock. The u.v.-lamp intensity was determined by using the inactivation dose ($D_{1\%}$ 230 ergs/mm^2) measured by Setlow and Boyce for phage $T4$ as a standard reference[10]. For calibration of the powerful lamp it was found convenient to use phage MS2 (this strain was kindly given to us by Dr. R. L. Sinsheimer, Pasadena, in 1963) as a reference which is more u.v.-resistant than $T4$ ($D_{1\%}$ 2760 ergs/mm^2). This phage was added at a low concentration to a $T4Bo^r$ suspension.

Extraction of DNA from phage. After treatment of the phage with Duponol the DNA was extracted by phenol. Our technique has already been described in detail [11]. After dialyzing the DNA for 24 hr against NCE-buffer $10^{-5}M$ spermine was added (NCES). Usually this stock solution contained about 80 μg DNA/ml as measured by the absorbance at 260 nm (absorbance$^{(1\,cm)}_{(260\,nm)} \times 52 \mathrel{\widehat{=}} \mu g$ $T4$-DNA/ml). To avoid breakage of the DNA molecules by shearing only pipettes with cut off tips and an interior diameter of more than 0·9 mm have been used for all further steps of handling DNA.

Sucrose density-gradient centrifugation. Zone centrifugation of the DNA was carried out in concentration gradients of 5–20% sucrose. Linear gradients were generated by the mixing chamber designed by Britten and Roberts[12]. On top of a gradient (4·6 ml) of neutral sucrose (5–20% w/v sucrose (ultrapure for gradient centrifugation, Serva, Heidelberg) in 1M NaCl, 0·01M Tris-HCl-Buffer, $10^{-3}M$ EDTA, pH 7·5) 0·2 ml of 5% neutral sucrose was added and then 4 μg of DNA in 0·2 ml NCES-buffer overlayered. Alkaline sucrose gradients (5–20% w/v sucrose in 0·9M

NaCl, 0·1*M* NaOH, $10^{-3}M$ EDTA, pH 12·5) were overlayered with 0·2 ml of 0·5*M* NaOH and 0·2 ml DNA. After running for 7 min at 5500 rev/min the neutral gradients were centrifuged 3 hr and the alkaline gradients 3·5 hr at 25,000 rev/min in the SW 50L swinging bucket rotor of a model L2-65K Spinco ultracentrifuge at 20°C. After centrifugation the cellulose nitrate tubes were placed on a rubber support giving a tight connection between tube and a steel cannula. Then the tube was pierced at the bottom by passing a needle through the cannula, and the emerging drops collected in a way that 3 drops were used for measurement of the ^{14}C-radioactivity and the following 5 drops discarded, etc. By employing this technique we received usually 49 fractions for the neutral, and 47 fractions for the alkaline gradients.

Measurement of radioactivity. The fractions obtained after zone centrifugation were collected in 20 ml glass vials fitted with glass-fiber disks of 2 cm dia. (Schleicher & Schüll, Germany, grade No. 6). Air-dried disks were treated with 1 ml of 10% TCA, 10 ml absolute ethanol, and 10 ml acetone, dried again and immersed in 10 ml of toluene scintillation mixture (0·1 g POPOP and 4·0 g PPO per liter of toluene), and counted in a scintillation counter (Mark I, Nuclear Chicago). The data are plotted as a percentage of total input radioactivity vs. fraction number. The relative molecular weight of the DNA species was calculated from the distances (D) sedimented, applying the relationship proposed by Burgi and Hershey [13] for linear molecules:

$$\left(\frac{D_2}{D_1}\right) = \left(\frac{M_2}{M_1}\right)^{0.35}$$

In some profiles the effect of radiation on the sedimentation properties of the DNA was estimated by calculation of the percentage DNA remaining at the molecular distribution of the unirradiated control measuring the areas of the curves with an integrator.

RESULTS

1. *Sedimentation behavior of DNA extracted from u.v.-irradiated unsubstituted phage*

Figure 1 shows the distribution of native *T*4 DNA sedimenting in a neutral sucrose gradient. The sharply defined peak of the control DNA extracted from unirradiated phage represents a homogeneous population of molecules. When extremely high u.v.-doses in the range of 80 phage lethal hits (PLH) (corresponding to about $3{\cdot}5 \times 10^3$ ergs/mm^2) were given to the phage a small portion of faster sedimenting molecules were observed in addition to the main peak. They probably correspond to molecules in which intramolecular and DNA-protein cross-links have occurred *in vivo* resulting in a change of coiling and sedimentation properties of DNA. It is evident, however, that under the experimental conditions shown in Fig. 1 no material appears which sediments slower than the mass of the DNA molecules, i.e. no detectable double-strand breaks are produced in normal phage by u.v.-light of 2537 Å.

DNA extracted from control phage as well as from phage which received 40 PLH (corresponding to about $1{\cdot}7 \times 10^3$ ergs/mm^2) was denatured and analyzed in an alkaline sucrose gradient and is shown in Fig. 2. Comparing the distances from the meniscus travelled by native and denatured control DNA respectively, one has to keep in mind, that for practical reasons the alkaline gradients were centrifuged for 210 min instead of 180 min. From position and width of the control curve shown in Fig. 2 it follows that

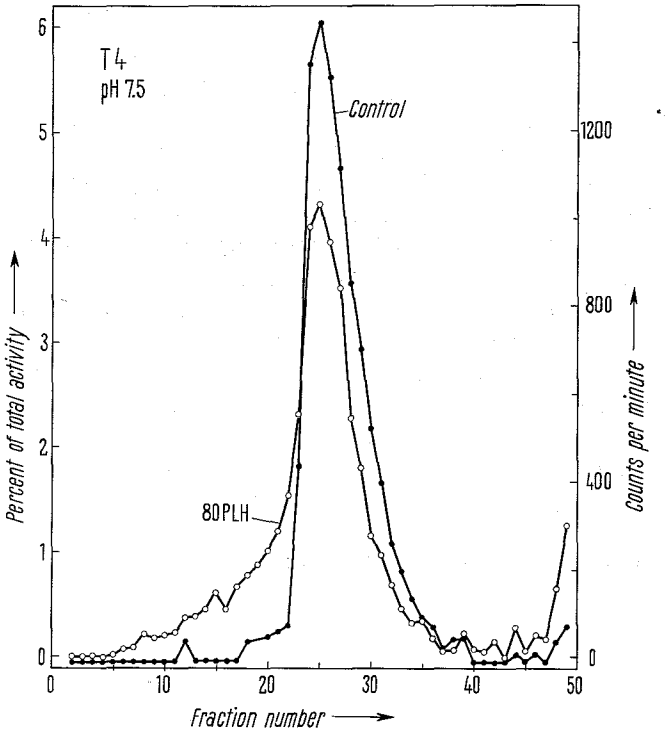

Fig. 1. Sedimentation patterns of native DNA phenol-extracted from phage $T4Bo^r$ before and after u.v.-irradiation (2537 Å) with $3{\cdot}5 \times 10^3$ ergs/mm^2 (80 PLH). All preparations of native DNA were sedimented in a neutral sucrose gradient (5–20% w/v) for 3 hr at 25,000 rev/min in the Spinco SW50L swinging bucket rotor at 20°C.

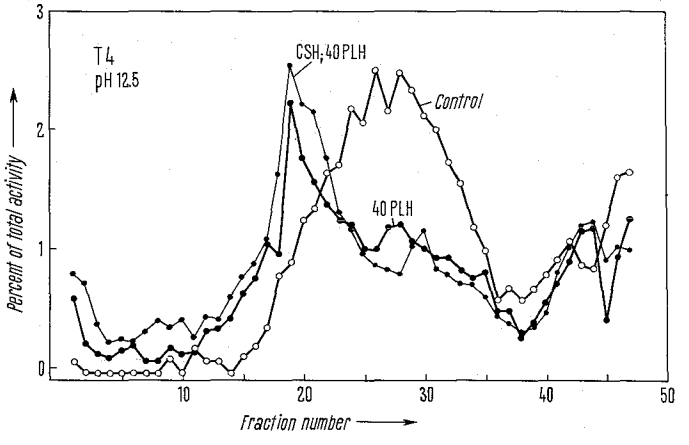

Fig. 2. The effect of u.v.-light ($1{\cdot}7 \times 10^3$ ergs/mm^2; 40 PLH) on the sedimentation pattern of alkali-denaturated DNA of phage $T4Bo^r$. Phage were irradiated in the absence and presence of 0·01 *M* cysteamine (CSH). All preparations of denatured DNA were sedimented in an alkaline sucrose gradient of pH 12·5 for 3·5 hr at 25,000 rev/min under the same experimental conditions as is described in the legend of Fig. 1 for native DNA.

DNA molecules from unirradiated *T*4Bo^r sedimenting in alkaline sucrose are broken into fragments to some extent. The population of molecules is much less homogeneous regarding their length and has a reduced average weight. After an u.v.-dose of 40 PLH (corresponding to about $1{\cdot}7 \times 10^3$ ergs/mm^2), a new species of molecules is observed (Fig. 2) which comprises 35 per cent of the whole population of DNA molecules and sediments faster than unirradiated DNA. This fraction of DNA can be attributed to intramolecular cross-linked molecules, whose strands cannot be separated under denaturing conditions.

Since the effect of cysteamine on the radiation damage due to BU-incorporation will be studied in Section 6 the result of a control experiment with normal DNA is included in Fig. 2. It is evident from the sedimentation pattern that 0·01 *M* cysteamine (Cysteamine-base, Fluka, Switzerland) present during u.v.-irradiation of the phage has no effect on the sedimentation behavior of unsubstituted DNA.

2. *Sedimentation behavior of native BU-DNA extracted from u.v.-irradiated phage*

Figure 3 gives the results of a series of experiments with DNA extracted from phage BU-*T*4Bo^r, which had been irradiated with increasing doses of u.v.-light. The control DNA from unirradiated BU-phage shows a sedimentation pattern similar to

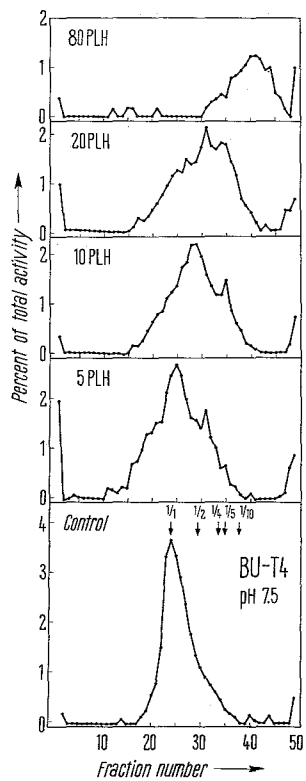

Fig. 3. Effect of increasing u.v.-dose on the sedimentation pattern of native BU-DNA isolated after irradiation of BU-substituted phage *T*4Bo^r. Arrows indicate the position of whole, half, quarter, etc. molecules.

that exhibited by the DNA of normal phage given in Fig. 1, indicating homogeneity of the molecules regarding their molecular weight.

After u.v.-irradiation with doses of 5 PLH (corresponding to about 100 ergs/mm^2) there is a decrease of material sedimenting at the position of the control. There appears a fraction of faster sedimenting material probably cross-linked molecules (intramolecular and DNA-protein cross-links). In addition a slowly sedimenting peak was observed. With increasing u.v.-dose on the phage the latter peak increases in size and is shifted towards the meniscus indicating an increased proportion of BU-DNA fragments with a smaller molecular weight. From the observed sedimentation behavior of BU-DNA in a neutral sucrose gradient we conclude, that during u.v.-irradiation of BU-phage the DNA is fragmented as a consequence of double-strand breaks.

3. *Comparison of the sedimentation behavior of native BU-DNA after u.v.-irradiation* in vivo *and* in vitro

In order to obtain information on the question of whether double-strand breaks observed after DNA extraction of irradiated phage are produced by the procedure of DNA separation from the phage head, we irradiated BU-DNA after phenol extraction of unirradiated phage with the same u.v.-dose as was given to the phage in the experiments shown in Fig. 3. It is evident from Fig. 4 that the double-strand breaks observed in our experiments with DNA from irradiated phage (Fig. 3) are not the consequence of phenol extraction. At a dose of about 430 ergs/mm^2 (20 PLH) the sedimentation behavior of BU-DNA irradiated in the free state, i.e. *in vitro*, results in a reduction of sedimentation velocity similar to that of BU-DNA u.v.-irradiated *in vivo*. The increased area of the curve representing DNA irradiated *in vitro* is due to a higher concentration of DNA in the tube. From comparison of the profiles shown in Fig. 4 we conclude that during u.v.-irradiation *in vivo* more double-strand breaks are produced than *in vitro*. This could be due to the different state of the DNA during irradiation, i.e. random-coil double-helix or 'condensed' conformation inside the protein head of the phage.

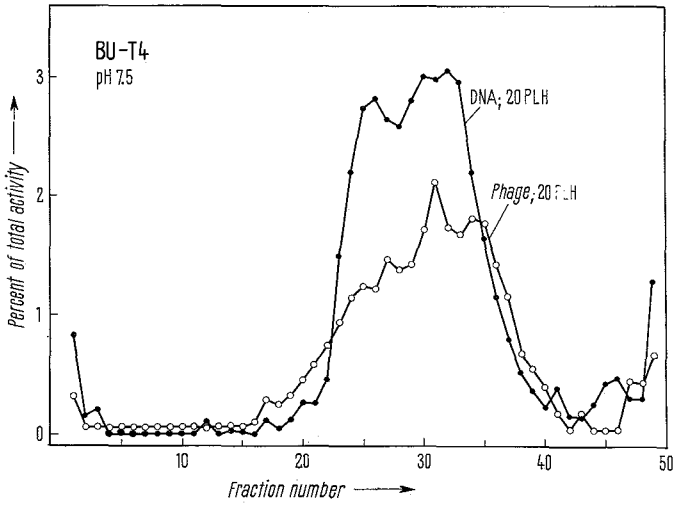

Fig. 4. Comparison of sedimentation patterns of native BU-DNA u.v.-irradiated (430 ergs/mm$_2$; 20 PLH) before and after isolation from BU-substituted phage $T4Bo^r$.

4. *Sedimentation behavior of denatured BU-DNA extracted from u.v.-irradiated phage*

The same DNA as was used for the experiments described in Section 2 (neutral sucrose gradients, Fig. 3) was also taken simultaneously for alkaline sucrose gradients at pH 12·5. The results are summarized in Fig. 5. It is evident from the data that: The sedimentation behavior of the DNA extracted from unirradiated BU-*T*4Bo^r is comparable to the sedimentation rate of unsubstituted DNA (Fig. 2) concerning shape and position of the band. With increasing u.v.-dose a characteristic shift of the DNA-band towards the meniscus is observed. This shift is proportional to the dose. The altered sedimentation rate of the denatured *DNA* irradiated *in vivo* indicates a decrease

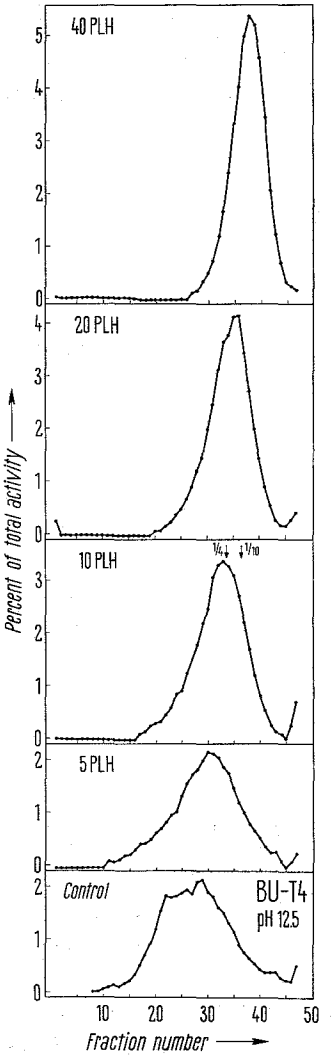

Fig. 5. Effect of increasing u.v.-dose on the sedimentation pattern of denatured BU-DNA isolated after irradiation of BU-substituted phage *T*4Bo^r. Arrows indicate the position of quarter and tenth molecules.

in molecular weight, which probably is due to an increase in single-strand breaks proportional to the u.v.-dose.

5. *Comparison of the sedimentation behavior at alkaline pH of BU-DNA after u.v.-irradiation* in vivo *and* in vitro

For the same reason as discussed in Section 3, i.e. to give an answer on the question of whether breaks are produced by extraction of the DNA, the sedimentation behavior of DNA irradiated inside the phage head was compared with the sedimentation rate of DNA irradiated after isolation with identical u.v.-doses. The results shown in Fig. 6 indicate that the marked decrease in molecular weight of single-stranded BU-DNA observed is not due to breakage of the strands during phenol extraction of the irradiated molecules. The observed difference between the profiles shown indicates that even more single-strand breaks are observed if the DNA is irradiated as a random-coiled helix in the free state. This is not surprising since it is reasonable to assume that under such conditions photochemical effects should be different [14].

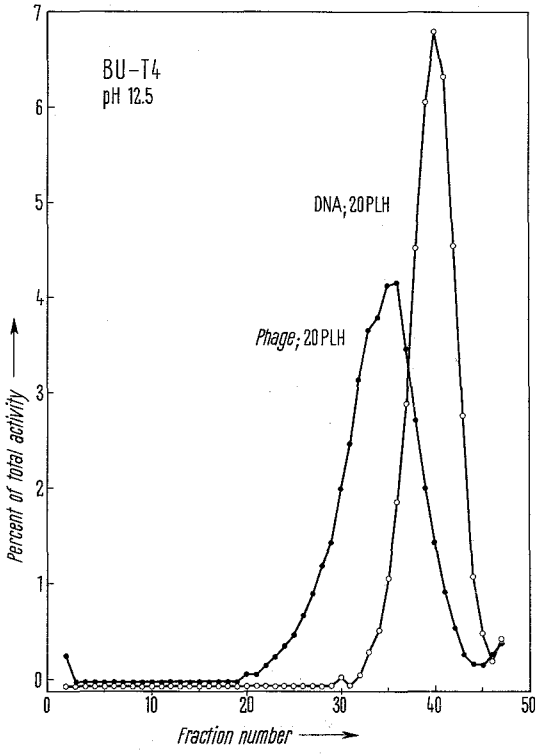

Fig. 6. Comparison of sedimentation patterns of denatured BU-DNA u.v.-irradiated (430 ergs/mm^2; 20 PLH) before and after isolation from BU-substituted phage $T4Bo^r$.

6. *Influence of cysteamine on u.v.-induced strand breaks in BU-DNA*

Figure 7 gives the results of experiments which have been performed with the aim of elucidating the influence of protective compounds on the u.v.-damage due to BU-incoroporation. Since the sedimentation behavior of unirradiated control DNA and

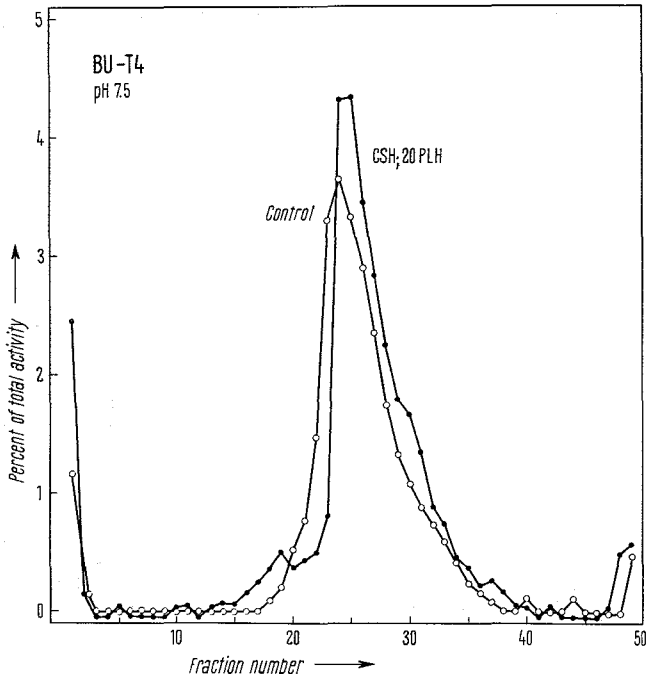

Fig. 7. Effect of 0·01*M* cysteamine (CSH) on the sedimentation pattern of native BU-DNA. Before isolation of the DNA the BU-substituted phage *T*4Bo^r received an u.v.-dose of 10^3 ergs/mm^2 (20 PLH) in the presence of the compound.

BU-DNA which received about 10^3 ergs/mm^2 (20 PLH) is identical, it is obvious from these data that the presence of 0·01*M* cysteamine during u.v.-irradiation of phage abolishes the decrease in sedimentation rate observed with native BU-DNA irradiated in the absence of cysteamine. We conclude that cysteamine effectively prevents double strand breaks which appear in BU-DNA in the absence of this compound (Fig. 3). This radioprotective effect, however, could not be observed if the DNA was sedimented in an alkaline sucrose gradient, i.e. after separation of the strands (Fig. 8).

DISCUSSION

The significant results of our experiments on u.v.-irradiated phage BU-*T*4Bo^r presented above will be summarized and discussed below: (i) In unsubstituted native DNA intramolecular and DNA-protein cross-links will occur upon u.v.-radiation *in vivo*, i.e. upon irradiation of entire phages. After application of a radiation dose of $3{\cdot}5 \times 10^3$ ergs/mm^2 (80 PLH) about 25 per cent of DNA appears as rapidly sedimenting material (Fig. 1). The sedimentation behavior of this DNA can be explained by the fact that within a single *T*1-DNA molecule intramolecular cross-links are formed *in vivo* due to dimeric photoproducts between groups of non-complementary strands brought into juxtaposition. Such molecules are supposed to show a more strongly folded structure and, hence, also a higher sedimentation constant than the random coiled helix of undamaged DNA. The influence of DNA-protein cross-links on the coiling of this DNA cannot be excluded by our experiments.

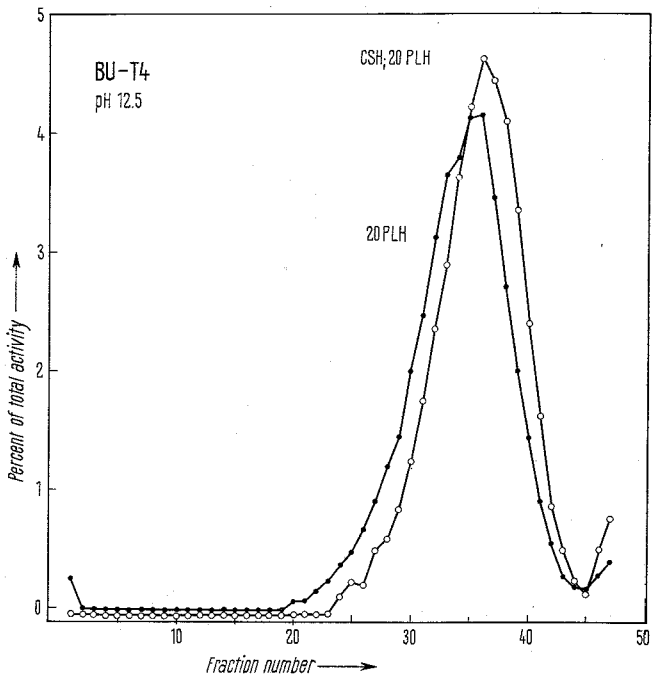

Fig. 8. Effect of 0·01 *M* cysteamine (CSH) on the sedimentation pattern of denatured BU-DNA. Before isolation of the DNA the BU-substituted phage *T*4Bo^r received an u.v.-dose of 10^3 ergs/mm^2 (20 PLH) in the presence of the compound.

(ii) In unsubstituted denatured DNA we also observed cross-links. These must necessarily be 'interstrand cross-links' which prevent the two DNA strands from being completely separated. The same molecular changes, measured by zone centrifugation, were recently described by other authors [15, 16] for denatured DNA of phage *T*2 and *T*4. The u.v.-doses applied in their experiments are identical to ours. Following an u.v.-irradiation with $1{\cdot}7 \times 10^3$ ergs/mm^2 (40 PLH) (Fig. 2) we observed an additional peak representing 35 per cent of the entire DNA, whose molecular weight is about 2·5 times higher than that of the control. Considering the small number of crosslinks produced per unit of dose, the biological importance of these molecular changes has to be called in question. (As to the effects of u.v.-light on the DNA-structure, reference should be made to the detailed publication of Smith [14].)

(iii) Chemical compounds of the cysteine–cysteamine group are known to provide effective protection from ionizing radiation [2]. However, these substances do not protect the biological activity of unsubstituted phage *T*1 [3], *T*4Bo^r [4], *T*2, *T*4, *T*4x (Hotz, unpublished results), and ϕX-174 [17] with respect to u.v.-radiation. The formation of cross-links in DNA after the irradiation of unsubstituted phage is likewise not influenced by the presence of 0·01 *M* cysteamine.

(iv) While molecular breaks in native DNA could not be detected after u.v.-irradiation of unsubstituted phage even at high doses, the DNA of BU-phage forms fragments of low molecular weights.

(v) Even high u.v.-doses to not produce single-strand breaks in unsubstituted phage. Contrary to that, we could detect by alkaline zone centrifugation the occurrence of

such breaks in denatured BU-DNA after u.v.-irradiation of phage with relatively low doses.

(vi) A discussion of the low effectiveness of u.v.-radiation in producing breaks in BU-DNA is only possible when considering that the production of breaks is accompanied by cross-links in the phage DNA. Therefore, it seems justified to assume two opposing processes with respect to sedimentation. Thus, a much higher number of broken molecules could be expected, if cross-links would not prevent the separation of two or more fragments. It is known that more cross-links occur in BU-DNA, as compared to normal unsubstituted DNA[14, 18].

With respect to the origin of the strand breaks in BU-DNA, a mechanism was proposed recently[4] the photochemical reactions of which start from an excited BU-molecule, and via radical reactions cause damage to the deoxyribose of DNA. These reactions finally can result in breaks of the DNA strands and in damage to other bases. If the enhanced sensitivity of u.v.-irradiated BU-phage is primarily caused by strand breaks, the ratio of breaks to lethal hits should be about unity. However, our findings revealed a much lower ratio. This result can be explained by the assumption that apart from breaks other lethal events, e.g. base-damage, occur in BU-DNA of phage *T*4Bo^r, which are produced with much higher yield.

(vii) Due to the presence of 0·01*M* cysteamine during u.v.-irradiation DNA of phage BU-*T*4Bo^r is protected from double-strand breaks measured in a neutral sucrose gradient. It is most interesting that the number of single-strand breaks visible in the alkaline gradient is not changed by the protective substance.

A quantitative analysis of the described processes would call for a more accurate determination of the number of cross-links as well as single- and double-strand breaks than we were able to conduct at the time with the method described above. However, setting apart this statement, we can conclude from our investigations that cysteamine is a substance which offers an effective radiation protection with respect to damages in phage DNA caused by BU and u.v.-irradiation. This applies to damage resulting both in a loss of plaque-forming ability, probably due to base damage, and in double-strand breaks of the BU-DNA. It was pointed out that a correlation might exist between the protective effect of cysteamine with respect to BU-damage and the ability of the host cell to reactivate radiation damage in BU-phage[4, 17]. This hypothesis was recently supported by experimental evidence furnished by Radman and collaborators[19].

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