Compounds of the Cysteine-cysteamine Group and their Influence on Infectivity, Strand Breaks and Base Damage in Gamma-irradiated DNA of Coliphage ΦX-174

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Single- and double-stranded (RF) DNA of page ΦX-174 were ^{60}Co-gamma irradiated. Dependence of radiosensitivity on the concentration of cysteamine and cystamine was measured. Radioresistance of DNA *in vivo* (phage particles) as well as of DNA *in vitro* (infectious DNA) increase between 10^{-4} and 10^{-1} M of superprotective agents. The same relationship is observed when the DNA is in the dry and in the wet state. By fractionating radiation damaged RFI and radiation induced RFII by means of sucrose gradient sedimentation it was possible to measure the protective effect of cysteamine on lesions other than strand-breaks. A pronounced radioprotective effect of cysteamine on the production of single-strand breaks in RF–DNA was found (DRF 20). The convenience of the RF system for studying effects of radioprotective agents on the occurrence of different radiation induced lesions is discussed.

1. Introduction

During the last decade particles as well as infectious DNA of phage ΦX-174 have been of increasing interest to molecular radiobiology. Besides investigations of the influence of various temperatures on the radiosensitivity of ΦX-particles [1] and their infectious DNA [2, 3], the action of atomic hydrogen [4], elastic nuclear collisions [5] and of vacuum-UV [6] on these objects were studied in detail.

Although "superprotection" [7] by cysteamine of broth suspended phage ΦX-174 from ionizing radiation was already reported [8], detailed and systematic investigation of radioprotection by compounds of this group in the biological system of ΦX-174 is lacking. Recent publications on the production of breaks in single-stranded ΦX-DNA [9] showed a reduction of these breaks when gamma-irradiated in the presence of cysteamine.

A work undertaken recently on the fractionation of certain radiation induced lesions in ΦX-RF DNA [10] suggested the study of the action of cysteamine and cystamine on single- and double-stranded DNA of phage ΦX-174. The experiments to be described show the same relationship between D_{37} and concentration of the protective substance to hold for particles and for infectious DNA when suspended in a medium with a protein content of some 5%, as well as for DNA irradiated after freeze-drying. In addition to a protective effect of cysteamine on lesions other than strand-breakage the number of radiation induced single-strand breaks of RF-DNA is reduced with an unexpectedly high efficiency.

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2. Material and Methods

The type and origin of the strains of phage ΦX-174 and of their different hosts were already described [10]. The preparation of ^{3}H-labelled RF-DNA, the technique of analytical sucrose-gradient centrifugation and of the DNA assay as well as of aerobic ^{60}Co-gamma irradiation were according to standard procedures [10–14]. Bacto nutrient broth (NB) was obtained from Difco, Detroit, USA, and cysteamine·HCl as well as cystamine·2 HCl from Calbiochem, Los Angeles, USA. The solutions were prepared using deionized and double-quartz-distilled water.

3. Results

3.1. Dependence of radiosensitivity on the concentration of cysteamine-cystamine

Fig. 1 shows the inactivation kinetics of single-stranded DNA from phage ΦX-174 when gamma-

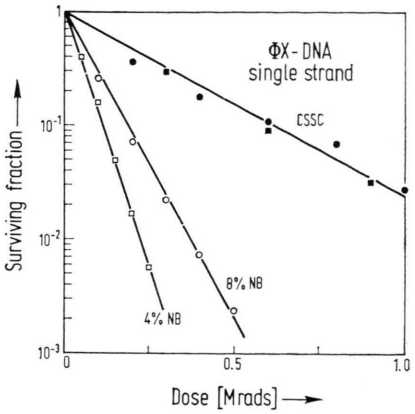

Fig. 1. Surviving fraction of infectious DNA isolated from phage ΦX-174. DNA was suspended in 4 per cent and 8 per cent Difco-nutrient broth (NB), respectively, and irradiated with a ^{60}Co-gamma source under aerobic conditions. CSSC = irradiation performed in 4 per cent (■) and 8 per cent (●) NB, respectively, in the presence of 0.1 M cystamine·2 HCl.

irradiated in NB-solution of two different concentrations. The inactivation of the biological activity follows a simple exponential function. The D_{37}, however, increases by a factor of about 1.7 when the DNA is irradiated in 8 per cent instead of 4 per cent NB (the usual standard medium in radiation experiments with bacteriophage). Such dependence of the D_{37} on protein concentration beyond about 1 per cent is not observed with phage particles and was studied recently using double-stranded infectious DNA of phage T1 [14]. Fig. 1 also gives information about the protective effect of 0.1 M cystamine. We conclude from the data that at this

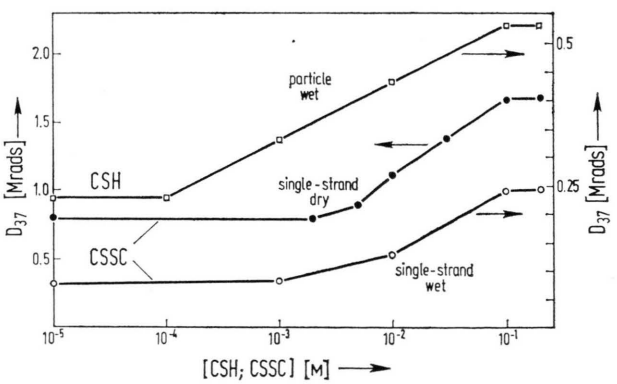

Fig. 2. Dependence of the inactivation dose (D_{37}) of ΦX-174 DNA *in vivo* (phage particle) and *in vitro* (single-stranded infectious DNA) on the concentration of cysteamine·HCl and cystamine·2 HCl, respectively. Particles are suspended in 4 per cent and single-stranded infectious DNA in 8 per cent NB, respectively. Infectious DNA was dried from 4 per cent NB.

concentration of cystamine a plateau of the D_{37} of infectious DNA is reached, which is independent of the concentration of organic material in the NB-solution. Analogous observations were already published for other types of phage particles [15]. Fig. 2 shows the relation between D_{37} and the concentration of radioprotective substance. From these results the following conclusions can be drawn:

a. No protective effect of cysteamine or cystamine can be observed at concentrations below 10^{-4} M. Beyond this value, the D_{37} increases with concentration and the curve tends to reach a plateau around 10^{-1} M.

b. The general shape of the curves is the same for ΦX-particles irradiated in the wet state and for isolated infectious DNA irradiated in the wet or in the freeze-dried state. It should be stressed that for the latter system the plateau of the curve is also at a molarity of 10^{-1} of protective substance.

c. At the plateau region the dose-reduction-factor (DRF) is 2 for the particle as well as for dried infectious DNA, and 3.8 for infectious DNA irradiated in the wet state.

3.2. Effect of cysteamine on the production of single-strand breaks in RFI-DNA

Fig. 3 shows the sedimentation profiles of RFI-DNA irradiated at increasing doses of ^{60}Co-gamma rays in the presence of 0.1 M cysteamine. The sedimentation direction in the neutral sucrose gradient is from right to left. It is evident that the transfer of material from the faster sedimenting RFI-band to the RFII-band increases with increasing radiation dose absorbed. This conversion can be explained by radiation-induced single-strand breaks. For comparison the profiles of two controls are drawn (broken lines) showing the corresponding sedimentation behaviour of RFI-DNA irradiated in the absence of the radioprotective compound. We conclude from these results that cysteamine reduces very efficiently the production of radiation-induced single-strand breaks.

Fig. 4 shows the fraction of RFI molecules irradiated in the absence and presence of 0.1 M cysteamine (4 per cent NB), respectively and still sedimenting in the RFI-band. The data are obtained from Fig. 3. From the slope of the curve marked CSH it is calculated that at about 1050 krads 37 per cent of the total number of irradiated molecules are without a single-strand break. The D_{37} is about

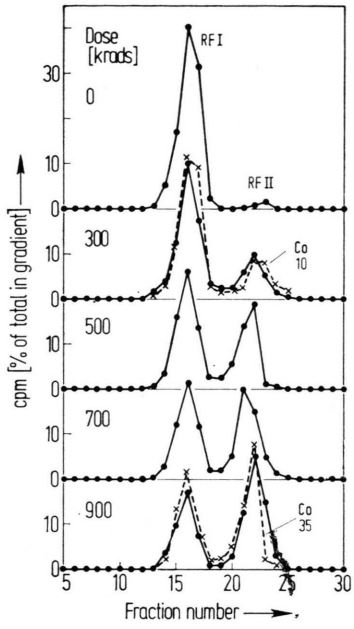

Fig. 3. Neutral sucrose-gradient sedimentation of RFI-DNA suspended in 4 per cent Difco-nutrient broth and gamma-irradiated in the presence of 0.1 M cysteamine·HCl. The sedimentation profile is represented by plotting the radioactivity [cpm] of the fractions as percentage of the total cpm in the gradient. Direction of sedimentation is from right to left. 0.5 ml DNA solution irradiated at 0 °C was layered onto top of 10 ml sucrose-gradient (5—20% w/v) and sedimented at 25,000 rpm in a SW41 swinging bucket rotor of a Spinco L2-65 ultracentrifuge at 10 °C for 17 hours. Twenty drop fractions were collected using three for measuring the radioactivity. For comparison the profiles of two control runs (Co) using RFI-DNA irradiated to 10 and 35 krad, respectively, in the absence of cysteamine are drawn (broken lines).

50 krads when the DNA is irradiated in the absence of cysteamine, *i. e.* under these experimental conditions cysteamine provides a protection factor of 21.

3.3. Effect of cysteamine on the infectivity of radiation-damaged RFI- and radiation-induced RFII-DNA

After irradiation of RFI-DNA in the presence and in the absence of 0.1 M cysteamine, respectively, the radiation induced RFII-DNA was fractionated by sucrose-gradient centrifugation from the material still sedimenting in the RFI-band (Fig. 3). The fractions containing RFI- and radiation induced RFII-DNA, respectively, were both adjusted to the same concentration, their biological activities assayed thereafter and compared to the infectivity of unirradiated RFI-DNA samples.

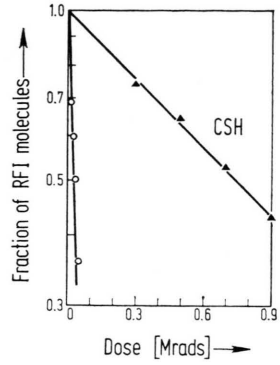

Fig. 4. Fraction of RFI molecules not converted to RFII by the occurrence of single-strand breaks produced by gamma-irradiation in the absence and presence of 0.1 M cysteamine. The data are taken from profiles such as shown in Fig. 3.

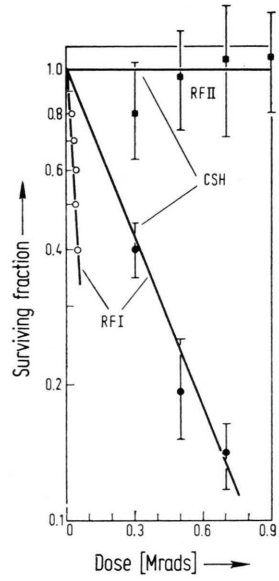

Fig. 5. Surviving fraction (plaque forming ability) of the products of RFI-DNA produced by gamma-irradiation in absence and presence of 0.1 M cysteamine, respectively. Neutral sucrose-gradient sedimentation was performed as described in legend to Fig. 3. At the end of the run the fractions from the left side of the RFI-peak containing RFI-DNA only and the fractions from the right side of the RFII peak containing RFII-DNA only were collected and both samples adjusted to the same concentration (radioactivity). After infection of *E. coli* K12-Spheroplasts the biological activity of both DNA samples (radiation damaged RFI and radiation induced RFII) was compared with the infectivity of unirradiated RFI-DNA.

It is evident from Fig. 5 that the biological activity of the molecules sedimenting in the RFI-band shows an exponential decrease when assayed in spheroplast prepared from *E. coli* K12 (ability of host cell reactivation). The D_{37} is about 70 krads in

the absence and 350 krads in the presence of 0.1 M cysteamine. We assume that the protective effect is due to reduction of DNA lesions other than breaks, *i. e.* mainly base damage. Contrary to the radiation damaged RFI-DNA the biological activity of radiation-induced RFII-DNA is not significantly altered (as tested by variance analysis for the $p = 0.05$-level) in the presence of 0.1 M cysteamine and within the dose range studied. However, this corresponds to what was expected from the data of Stephan [10] on the inactivation kinetics of radiation-induced RFII-DNA since in the absence of cysteamine these molecules do not show any damage to the biological activity up to radiation doses of about 50 krads, *i. e.* single-strand breaks are not lethal in RF-DNA. Unfortunately, this is the highest dose, which can be employed with radiation induced RFII in the absence of cysteamine. Beyond this dose these molecules are disappearing from the RFII-band by marked change of their molecular configuration.

4. Discussion

It was shown in coliphage T1 [16] that under different experimental conditions the amount of superprotection provided by the thiol cysteamine and the corresponding disulfide (cystamine) is comparable within a few per cent. This observation is confirmed by our results given in section 3.1. which, in addition, show the phenomenon that radiosensitivity of free DNA in the absence of superprotecting agents is greatly influenced by the amount of protein present in the solution, *i. e.* the concentration of NB. This phenomenon was studied recently and discussed in detail for infectious T1-DNA [14] but was not observed with phage particles. The extent of radioprotection on infectivity provided by 8 per cent NB as compared with 4 per cent NB is increased by a factor of about 1.5. This holds for single-stranded DNA from ΦX-174 as well as for double-stranded DNA from phage T1.

Furthermore comparable values were obtained for the maximum protective effect of compounds of the cysteine-cysteamine group on infectivity of the two types of DNA mentioned. When suspended in 4 per cent NB the factors are about 5 for single-stranded ΦX-DNA and for RFI-DNA, and 7 for double-stranded T1-DNA [14]. For the time being we cannot offer a convincing explanation for this observation since a much greater influence on single-stranded DNA would be expected. However, multiplicity reactivation occurring in our assay system of single-stranded DNA might partly be responsible for the lack of a pronounced difference in this respect. This hypothesis could also explain the observation that the D_{37} of single-stranded and of RF-DNA is nearly equal in the absence [10] as well as in the presence of compounds of the cysteine-cysteamine group.

Any discussion of the mechanism by which cysteamine-cystamine protects DNA (*c. f.* [17]) requires elucidation of the way of interaction of protective compound with DNA. Our experiments (Fig. 2) show a similar concentration-effect dependence to exist for the influence of cysteamine on radiosensitivity of single-stranded DNA in the wet and in the dry state. This result suggests a strong interaction between the protective compound and the DNA occurring in the wet state and lasting for the period of freeze-drying. One effect of such interaction might be the chelating behaviour of cysteamine and the formation of ternary complexes between DNA-metal-cysteamine. Furthermore, these results strongly support the hypothesis that compounds of the cysteine-cysteamine group are acting mainly on DNA radicals.

Systems of small supertwisted rings of double-stranded DNA offer an ideal technique for testing the influence of protective substances on the production of non-break-damage as well as on the occurrence of single-strand breaks (ssb) without subjecting the DNA to any physical or chemical treatment in order to denaturate the molecule. This is of special interest since the occurrence of strand breaks at labile sites in radiation damaged DNA have been reported, *e. g.* by alkaline treatment [18, 19]. These findings have to be considered when the unusually high protective effect of cysteamine (DRF 21) on the occurrence of radiation-induced ssb in RF-DNA is discussed. However, this effect is not unexpected and our observation is in agreement with data published recently on phage T4 and T7 [20–23]. In the presence of compounds of the cysteine-cysteamine group the contribution of double-strand breaks to the lethal event increases, *i. e.* all those radical reactions are strongly suppressed which are responsible for indirect radiation effects and leading to the occurrence of single-strand breaks – in addition to damage to bases and sugar as well as cross-link production.

From the Table the relation of the number of lethal events to the number of single-strand breaks produced in RFI-DNA can be calculated. This ratio

Table. Radioprotective effect (DRF, D_{37}) of cysteamine (CSH) against inactivation of infectivity and production of single-strand breaks in different types of infectious DNA from phage ΦX-174.

Type of DNA	CSH [0.1 M]	Type of damage	D_{37} [krads]	Protection factor (DRF)
single-strand	−	loss of Infectivity	70	5.3
	+		370	
RFI	−		70	5
	+		350	
	−	single-strand breaks	50	21
	+		1050	

is about unity in the absence of cysteamine. Obviously, the D_{37} for altering RFI by ssb cannot be much less than the value of 50 krads given in the table since every ssb changes the configuration of RFI to RFII. Since radiation induced RFII is biologically fully active [10], we conclude from these results that most of all ssb produced in RFI and leading to the formation of radiation induced RFII are not lethal events in RF-DNA of phage ΦX-174. In the presence of cysteamine, however, the above mentioned ratio changes to 1 : 0.3, *i. e.* the protective effect of cysteamine is much greater on the production of ssb than on other lesions, *e. g.* damage to bases and/or deoxyribose. It seems to be plausible that in the presence of radioprotective compounds the spectrum of lesions which contribute to the biological inactivation is markedly altered. This phenomenon of the distribution of the spectrum being widely influenced by a radical scavenger was studied recently in double-stranded infectious DNA of phage T1 [14].

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