KFK-157

KERNFORSCHUNGSZENTRUM

KARLSRUHE

Februar 1963

KFK 157

Institut für Strahlenbiologie

Temporary Radiostability in Extracellular Bacteriophages after Trapping of Molecules of the Cysteine-Cysteamine Group

Gerhart Hotz

KERNREAKTOR
Bau- und Betriebs-Gesellschaft m. b. H.
Verwaltung der Zentralbücherei
25. Juli 1963

KERNREAKTOR

BAU- UND BETRIEBS-GESELLSCHAFT M.B.H.

KARLSRUHE

Reprinted from
INTERNATIONAL JOURNAL OF RADIATION BIOLOGY, Vol. 6, No. 2, p. 143, February 1963

Temporary radiostability in extracellular bacteriophages after trapping of molecules of the cysteine-cysteamine group

GERHART HOTZ
Institut für Strahlenbiologie, Kernforschungszentrum Karlsruhe, Germany

(*Received* 20 *September* 1962)

Ten different strains of bacteriophages are checked for a new phenomenon tentatively designated as ' phage-SH complex '. The phages are incubated in nutrient broth solution to which 0·5 M cysteine or cysteamine is added. The x-irradiation, however, is performed with the phages in an anoxic medium in which the sulphydryl concentration is reduced below that concentration sufficient for protection.

It is observed that some phages are able to acquire a temporary radioresistance. The effect of oxygen and of an SH-blocking compound on the phage-SH complex was investigated and related to phage structure. The protective mechanism is discussed in the light of current hypotheses.

1. INTRODUCTION

The well-known differences in structure and permeability of the protein membrane of bacteriophages may at least partly be responsible for the fact that phages of different strains protected by cysteine act quite differently in the presence of oxygen (Hotz and Müller 1960). It has been suggested that sulphydryl compounds present in the suspending medium might reverse radiation-induced damage in the phage by donating hydrogen atoms for restoration of the biomolecule (Howard-Flanders 1960). For such a mechanism to occur, penetration of the protective molecules into the phage is necessary and should depend on the physical and chemical properties of the membrane of the phage. Recently, it has been observed (Hotz and Müller 1962, Marcovich 1962) that after incubation with sulphydryl compounds, extracellular bacteriophage T2 can acquire a certain degree of radioresistance (or radiostability) and, subsequently retain this state for a certain time, even in the absence of protective SH-compounds in the medium surrounding the phage particles.

In the light of recent hypotheses (Alexander and Charlesby 1955, Eldjarn and Pihl 1956, Howard-Flanders 1960) proposed for the protective mechanism of sulphydryl substances, it seemed worth-while to investigate the interaction between phage and sulphydryl compounds (Zimmer 1961) and the effect of the presence of oxygen, e.g. by using phage pre-treated with SH-compounds and irradiated in suspensions free from interference by the protective molecules dissolved in the phage suspension.

2. MATERIALS AND METHODS

Purified and concentrated suspensions of different strains of bacteriophages were diluted tenfold into 4 per cent Difco-nutrient broth, thereby avoiding most of the so-called indirect radiation effects. The actual phage titre per millilitre was usually 10^9, but in the experiments forming the phage-SH complex around 10^{12}. The SH-compound cysteine (Nordmark, Hamburg), or cysteamine

KERNREAKTOR
Bau- und Betriebs-Gesellschaft m. b. H.
Verwaltung der Zentralbücherei

(Fluka, Switzerland), was added to the broth solution in a concentration which gives maximum protective effect (Hotz and Müller 1962). The pH was adjusted to about 7. A special technique for forming phage-SH complexes will be described in § 3.1 of the results. Irradiations were performed in a plexiglass cup of 20 mm diameter and supplied with 0·6 ml of the phage suspension each. Equilibration of the suspension with purified nitrogen or oxygen was assured by blowing a jet of the gas onto the suspension before and during irradiation. The x-ray source was operated at 100 kvp with 0·5 mm plexiglass filter. The effective wavelength of the source was calculated as 1·4 Å, and the dose-rate approximately 250 kr/min. Some control experiments have been made with the more penetrating radiation from a 150 kvp x-ray beam (dose-rate about 10 kr/min) and a cobalt gamma-ray source (dose-rate approximately 7 kr/min). The radiation energy absorbed by the phage suspension was measured during irradiation by a soft-ray ionization chamber (Physikalisch-Technische Werkstatten, Freiburg, Germany) calibrated against a Baldwin–Farmer sub-standard ionization-chamber, and, in addition against the Fricke actinometer ($Fe^{+2} \longrightarrow Fe^{+3}$).

3. Results

3.1. *Forming of the phage-SH complex*

The data of a typical experiment with phage T4 are shown in figure 1. The lower curve represents the surviving fraction of T4 wild-type and of a T4 mutant strain in nutrient broth after irradiation with 100 kvp x-rays (△) or with gamma-rays (○). The upper curve (●) indicates the sensitivity of T4 wild-type phage pre-treated for 5 min at room temperature with 0·5 M cysteine, but diluted before anoxic irradiation to an actual molarity of the sulphydryl compound of 5×10^{-5}. This sulphydryl concentration, without the special pre-treatment technique, gives no protection at all (Hotz and Müller 1962). In addition to T4, we find this phenomenon (tentatively designated as ' phage-SH complex ') with some other phages. They are listed in the table together with the dose-reduction factor (DRF) achieved by irradiating the phages protected by 0·2 M cysteamine (CYA) present in anoxic broth. The standard error of the 37 per cent-dose for $p = 0{\cdot}05$ was calculated to be $\leqq 10$ per cent. Our results seem to indicate that, besides phage ØX174, only the T-even phages show this ' binding ' phenomenon. These latter phages differ markedly from the others in having a rigid protein membrane which can be ruptured by osmotic shock. Whether this structural difference is of importance for the phenomenon described and could, at least partly, be responsible for the ' binding ' or ' trapping ' of the sulphydryl molecules, was investigated through comparison with a shock-resistant mutant of T4 (called T4 Bo^r) kindly supplied to us by Professor Harm, Cologne. It was found that this phage, in contrast to the shock-sensitive wild-type phage, does not develop radiostability by binding the protective molecules (figure 1; ▲).

3.2. *The effect of oxygen on the complex*

The influence of oxygen on the ' acquired ' radioresistance was tested with a T2-cysteamine complex. This compound was chosen because of its higher stability against oxidation compared with the compound cysteine.

Equilibration of such a phage suspension with oxygen either before or during irradiation completely removed the protective effect afforded by the sulphydryl compound.

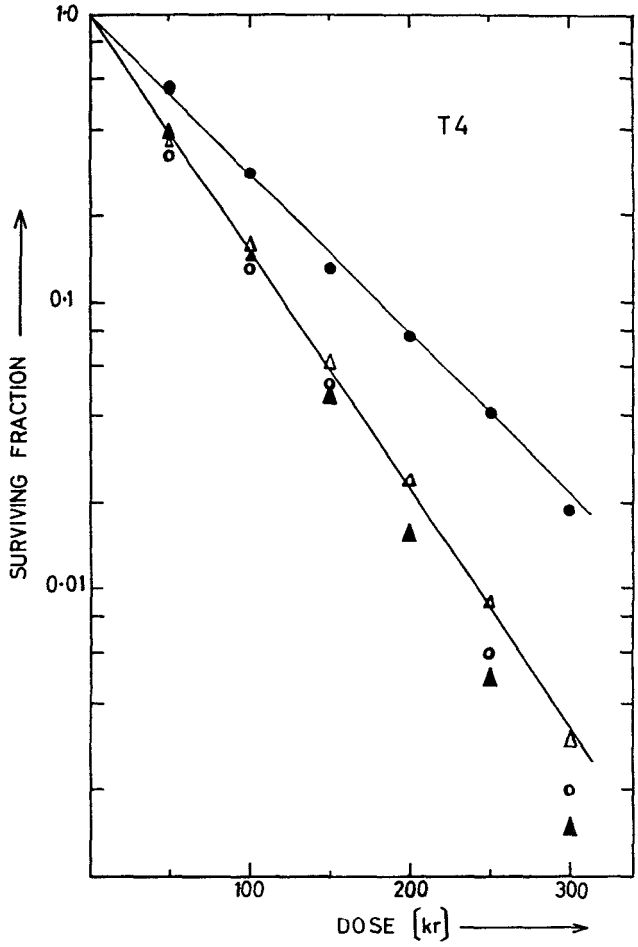

Figure 1. △ Surviving fraction of T4 phages after anoxic irradiation in 4 per cent Difco-nutrient broth with 100 kvp x-rays, or ○ with ^{60}Co gamma-rays (aerobic conditions); ● T4 wild-type phage; ▲ shock-resistant mutant T4 Bo^r after pre-treatment with 0·5 M cysteine (anoxic conditions).

Phage strain	DRF in 0·2 M CYA	Forming of phage-SH complex
T1	2·4	−
T2	2·9	+
T4	2·5	+
T4Bo^r	3·6	−
T5	3·0	−
T6	—	+
T7	2·4	−
P22	2·9	−
P22vir.	—	−
ØX174	2·5	+

The dose-reduction factor of ten different phage strains is listed in the second row. This value corresponds to the slope difference of complete survival-curves for phages x-irradiated in anoxic 4 per cent Difco nutrient broth with and without 0·2 M cysteamine (CYA) respectively present during irradiation. In the third row is listed the ability of the strains to form a phage-SH complex and to keep a temporary radiostability in a medium which is lacking a sufficient amount of protective sulphydryl molecules. The standard error of the 37 per cent doses for $p = 0{\cdot}05$ was calculated to be $\leqq 10$ per cent.

3.3. *Influence of N-ethylmaleimid (NEM) on the dose-reduction factor*

In order to test whether the protective molecules giving the acquired radiostability are ' trapped ' or ' bound ' or ' masked ' in some way, the action of the SH-blocker N-ethylmaleimid in the T4–T4Bo^r system was studied. This compound, which is believed to combine with freely-reacting —SH groups, was found to have no effect on the protective ability of sulphydryl molecules trapped by T4. The results of such experiments are given in figure 2. The dashed line (A) represents the survival-curve of irradiated T4 and T4 Bo^r in 4 per cent broth. The points indicated by triangles show the results of experiments with the shock-resistant, non-complex-forming phage T4 Bo^r, which is irradiated in nitrogen-saturated broth with M/100 cysteamine and M/100 NEM added. Prior to a tenfold dilution into the cysteamine–NEM mixture, the phage was treated

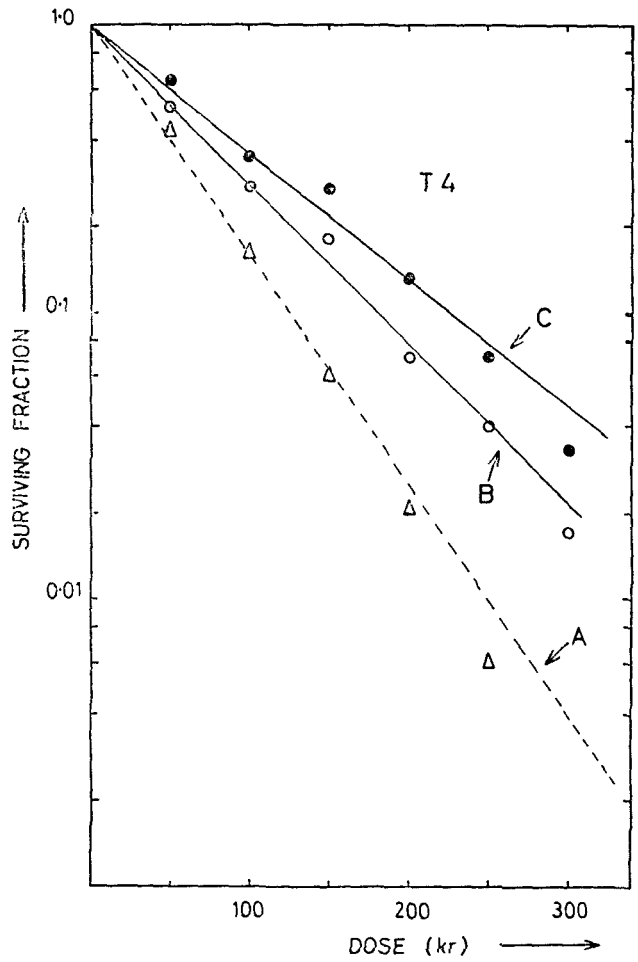

Figure 2. The dashed line A is the survival-curve of T4 phages irradiated in broth (same as in figure 1). △ T4 Bo^r and ○ T4 wild-type phage after pre-treatment with M/100 cysteamine and anoxic irradiation in broth with M/100 cysteamine and M/100 N-ethylmaleimid present. ● surviving fraction of T4 after irradiation in nutrient broth in the presence of M/100 cysteamine.

with a M/100 cysteamine–broth solution for 5 min. If the same experiment is performed under identical conditions with the shock-sensitive, complex-forming wild-type phage, one observes a radioprotection (figure 2, curve B) which is comparable with that achieved in an SH-complex-forming experiment (indicated in figure 1). For comparison, curve C shows the normal dose-effect curve of irradiated T4 phages protected by M/100 cysteamine present in the virus suspension.

4. Discussion

It is most interesting and informative that some bacteriophages can acquire a certain degree of radioresistance after incubation in a solution of sulphydryl compounds of the cysteine–cysteamine group. This ' acquired ' radioprotection is efficient although, in the medium surrounding the phage, the number of sulphydryl molecules is negligible. The mechanism of such an ' acquiring ' could be:

(1) A formation of mixed disulphides between biological —SH groups of the phage and analogue groups of the protective molecules, such as proposed by Eldjarn and Pihl (1956) for the radioprotective mechanism of compounds of the cysteine-cysteamine group.

(2) No binding of the sulphydryl compounds at all, but in some way a reducing action of these chemicals on sensitive sites of the phage not afforded by extensive bubbling of the phage suspension with pure nitrogen.

(3) A ' trapping ' of the protective molecules inside the phage membrane.

If explanation (1) were correct, there should be a complex formed between phage and the protective chemical. This is suggested by the finding that ^{35}S-labelled cysteamine is bound to the phage T2 and T4 (Hotz, unpublished results). Furthermore, the mixed-disulphide complex formed should be rather stable. The acquired radioresistance, however, diminishes continuously within a few hours. Two hours after having formed the complex, the DRF is diminished by about 25 per cent (Hotz, unpublished results). The velocity of dissociation of the complex into its components is higher at 37°c than at 0°c (Marcovich 1962). A splitting of the disulphide linkage could only be expected in an enzyme-containing system such as mammalian organs (Eldjarn and Pihl 1956). Furthermore, such a disulphide bond should not be affected by brief oxygen bubbling before irradiation, as was observed in our experiments. Our finding that phage T4 Bo^r, which is not able to acquire any radioprotection, binds under identical experimental conditions the same amount of labelled cysteamine as do T4 or T2 wild-type phage also disagrees with explanation (1). The main objection against explanation (1) however is the fact that only the T-even phages and ØX174 show this phenomenon of acquired radiostability. The observation that all phages so far tested—including the DNA single-stranded phage ØX174 (Hotz and Müller 1961)—are protected by sulphydryl compounds to about the same degree, suggests a similar protective mechanism for all phages. The probability is small that just a minor group of the bacteriophages is protected against ionizing radiations by the mechanism of mixed disulphides, but all the others by a different mechanism. This is especially unlikely for T4–T4 Bo^r system.

If explanation (2) were correct, the protection afforded by the sulphydryl compound should last as long as no new oxygen can diffuse to the reduced

phage sites. As a matter of fact, the acquired stability diminishes even if the slowly-oxidizing compound cysteamine is used and, in addition, the phage —SH complex is held under nitrogen atmosphere. Since only some phages can acquire this kind of radiostability, hypothesis (2)—which should apply, if correct, to all phages—is very unlikely. The anoxia theory, extensively discussed by Gray (1956), is undoubtedly of great importance for the protective mechanism in all higher cell-systems where the reducing sulphydryl compounds are acting by lowering the partial pressure of oxygen in the cell.

Of the explanations mentioned above, number (3) is the most likely one. If the protein membranes of bacteriophages differ in their permeability or (and) affinity to molecules from the suspending medium, great differences should also be found in the interaction between protective molecules and phages of different membrane structure. A dense membrane could influence the diffusion of the cysteine or cysteamine molecules out of the phage structure into the surrounding medium. Such a ' trapping effect ' would be in good agreement with our experimental findings. The effect could be absent in shock-resistant types of phages, even in a phage like T4 Bo^r so closely related to the T-even group. The observed resistance of cysteamine-pre-treated T4, and the absence of such a resistance in T4 Bo^r against the effect of equimolar addition of N-ethylmaleimid (figure 2), indicates a phage barrier different for both chemicals. A similar barrier against NEM was found in spores of *Bacillus subtilis* (Bridges 1961).

Concerning the different behaviour of the T-even and shock-resistant bacteriophages in trapping sulphydryl compounds, it may be of some interest to mention recently published data on polyamines acting as cations to neutralize the negatively-charged phosphorus of the T-even phage DNA (Ames and Dubin 1960). These polyamines have not been found in T1 and other osmotic shock-resistant phages. The authors suggested that the protein membrane of these latter phages is highly permeable to the polyamines, and that cations from the medium (like Mg^{++}) can displace the DNA-bound polyamines which are ' packed ' into the head of all phages by the host cell during phage-synthesis. Similar interaction between the sulphydryl compounds and the DNA of bacteriophages is possible. In this respect it is of importance to note that at the pH of 7 usually used in phage experiments, cysteine and cysteamine are positively charged as found in electrophoretic studies (Hotz, unpublished results).

It should be recalled here that Hollaender and Stapleton (1956) mentioned a similar effect of sulphydryl compounds bound to *Escherichia coli*. Thus, if radiation experiments with sulphydryl-protected biological objects are to be interpreted, it should be kept in mind that the radiosensitivity of viruses, bacteria and probably cells of higher organisms can be influenced by the fact that some structures can trap protective molecules. Such interaction between a biological structure and a protective molecule should occur *in vitro*, as well as in an *in vivo* system, where cysteine is a normal constituent of the cytoplasm. Although the ' binding ' of radioprotective sulphydryl compounds described here is not directly related to the protective mechanism by which these chemicals are acting, these studies on the interaction between phage and ' trapped ' protective molecules seem to be one approach to the better understanding of the interaction of x-irradiated phage with molecules of the cysteine–cysteamine group, and a step forward in drawing a distinction between current hypotheses. Concerning the protective mechanism by which *trapped* molecules of cysteine or cysteamine

are acting, we suggest a similar reaction as was proposed first by Alexander and Charlesby (1955) for a polymer system and recently for phage irradiated in a medium with sulphydryl compounds *present* (Howard-Flanders 1960), that is the restoration of the radiation-induced damage of the phage particle by giving a proton from the protective molecule to a short-lived DNA-radical.

ACKNOWLEDGMENTS

The author wishes to express his gratitude to Professor K. G. Zimmer for his stimulating interest throughout the course of this work, to Professor A. Catsch and Dr. A. Müller for many discussions and suggestions, and to Miss R. Mauser and Mrs. R. Walser for their technical assistance.

Dix différentes variétés de phages ont été étudiées en vue de la présence d'un nouveau phénomène désigné complexe de phage-SH. Les phages ont été incubés dans du bouillon nutritif en ajoutant 0,5 moles de cystéine ou de cystéamine. Mais ensuite on les a irradiés dans un bouillon anaérobique qui contenait une quantité de molécules de cystéines et de cystéamines respectivement, négligeable en ce qui concerne l'effet de protection. On a observé que quelques phages sont à même d'acquerir une résistence temporaire aux radiations. L'effet de l'oxygène et d'une liaison immobilisant le SH, sur le complexe a été analysé et l'influence de la structure des phages sur le phénomène ci-dessus étudiée. A la lumière de quelques hypothèses actuelles on a discuté le mechanisme de protection.

Zehn verschiedene Phagenstämme wurden auf das Vorhandensein eines neuen Phänomens untersucht, welches als Phagen-SH Komplex bezeichnet wird. Die Phagen wurden in Nährbouillon mit einem Zusatz von 0,5 molarem Cystein oder Cysteamin inkubiert, aber anschließend in einer anaeroben Bouillon bestrahlt, die eine für den Schutzeffekt vernachlässigbare Anzahl von Cystein- bzw. Cysteaminmolekülen enthielt. Es wurde beobachtet, daß einige Phagen in der Lage sind eine temporäre Strahlenresistenz zu erwerben. Die Wirkung von Sauerstoff, sowie die einer SH-blockierenden Verbindung auf den Komplex wurde untersucht, und der Einfluß der Phagenstruktur auf das beschriebene Phänomen studiert. Im Hinblick auf einige aktuelle Hypothesen wurde der in Frage kommende Schutzmechanismus diskutiert.

REFERENCES

ALEXANDER, P., and CHARLESBY, A., 1955, *Radiobiology Symposium*, Liège, 1954 (London: Butterworths Scientific Publications), p. 49.

AMES, B. N., and DUBIN, D. T., 1960, *J. biol. Chem.*, **235**, 769.

BRIDGES, B. A., 1961, *J. gen. Microbiol.*, **26**, 467.

ELDJARN, L., and PIHL, A., 1956, *Progress in Radiobiology* (London: Oliver & Boyd), p. 249.

GRAY, L. H., 1956, *Progress in Radiobiology* (London: Oliver & Boyd), p. 267.

HOLLAENDER, A., and STAPLETON, G. E., 1956, *Ciba Found. Symp. on Ionizing Rad. and Cell Metabolism.*

HOTZ, G., and MÜLLER, A., 1960, *Z. Naturf.* B, **15**, 450; 1961, *Ibid.*, **16**, 282; 1962, *Ibid.*, **17**, 34.

HOWARD-FLANDERS, P., 1960, *Nature, Lond.*, **186**, 485.

MARCOVICH, H., 1962, *Z. Naturf.* B, **17**, 23.

ZIMMER, K. G., 1961, *Studies on Quantitative Radiation Biology* (London and Edinburgh: Oliver & Boyd).