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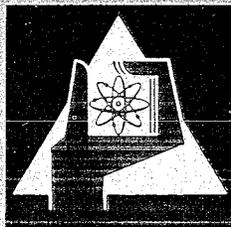
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Radiation Sensitivity of Bacteriophage DNA

II. Breaks and Cross-Links after Irradiation in vivo

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Radiation sensitivity of bacteriophage DNA II. Breaks and cross-links after irradiation *in vivo*

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Bacteriophage T1 suspended in aqueous solution of 0.165 M NaCl+0.1 M histidine was irradiated in a ^{60}Co - γ -source. The inactivation of the plaque-forming ability was followed, and the DNA was isolated after irradiation. The modifications of the DNA structure were studied by means of sedimentation analysis and viscosimetry. The number of the γ -induced double and single-strand breaks as well as the number of cross-links within the phage DNA and of cross-links between DNA and coat protein were calculated. It was shown, that per inactivated phage, 3.5 single-strand breaks, 0.17 double-strand breaks, 0.03 DNA-DNA-cross-links and 0.06 DNA-protein cross-links occurred.

1. Introduction

The absorption of an average energy of 60 eV, i.e. one primary ionization, is generally sufficient to cause inactivation of the plaque-forming ability (PFA) in bacteriophages with single-stranded DNA irradiated in nutrient broth (Ginoza 1967). If bacteriophages with double-stranded DNA are irradiated, the inactivation probability per primary ionization is much less than unity: mostly it lies between 0.05 and 0.2. The main reason for this difference, it is assumed, is that only a few specific lesions produced by radiation result in inactivation in the double-stranded phage, while the rest are ineffective. For a better understanding of these radiation effects, it is necessary, therefore, to have the completest possible description of the different physico-chemical alterations after irradiation and to relate them to the PFA.

In the present studies on the radiation-induced lesions in the phage, we restrict ourselves to lesions of the DNA structure, since DNA constitutes the most important radiation-sensitive target in phage (Ginoza 1967, Dertinger and Jung 1969). This applies in particular if the bacteriophages are irradiated in NB, i.e. in the presence of a high concentration of organic substances, where only the energy absorbed within the phage is effective. Among the molecular alterations of the DNA structure which may be significant for inactivation, breaks of the double helix and the single strand will be discussed in particular. Freifelder (1965, 1966, 1968) measured the number of double and single breaks in irradiated bacteriophages and compared it with the inactivation of PFA. In T5, T7 and λ -phage, half the inactivated phages had double breaks after irradiation in 10^{-3} M histidine; in T4 phage even 70 per cent showed double breaks. In addition, about ten times more single breaks than double breaks were counted. However, these values depended on the conditions of irradiation. It should also be taken into account that in the phage the DNA is densely packed, i.e. its physical state can be compared rather with a gel with only a low water content. If isolated DNA is irradiated in the gel state, a number of cross-links are observed besides the breaks

(Lett and Alexander 1961). Therefore, it was necessary to investigate the DNA of irradiated phages not only with respect to breaks but also to possible cross-links. By determining the coiling properties of DNA and the distribution of chain lengths, it was possible to deduce the frequency of such cross-links.

2. Material and methods

2.1. Bacteriophages and irradiation

The objects under investigation were bacteriophages T1. Preparation and purification of the lysates were performed by the method according to Hotz (1966). The phages (titre 2×10^{12} phages/ml.) were suspended in 0.165 M NaCl + 0.1 M histidine, pH 7, saturated with N_2 for 10 min in glass-stoppered tubes and irradiated in ice by a ^{60}Co gamma source (Gamma cell 220, Atomic Energy of Canada Ltd.) at 1.6 Mrads/hour.

2.2. Preparation of DNA samples

To remove the DNA released from burst phages also formed by irradiation, though to a small extent, the phage suspensions were treated after irradiation with 10 μ g/ml. deoxyribonuclease I (E.C. 3.1.4.5) (Worthington, B.C) for 30 min at 37°C. The phages were centrifuged at 14 000 g for 2 hours and resuspended in buffer. From this suspension, DNA was isolated by the methods described earlier (Mandel and Hershey 1960, Coquerelle, Bohne, Hagen and Merkwitz 1969). For better removal of residual coat protein, some DNA preparations were treated with trypsin (0.01 per cent in 0.08 M tris buffer, pH 7.8) for 16 hours at 20°C (Weinert and Hagen 1968) resulting in a reduction from some 3 to 1 per cent protein. The denaturation of DNA was carried out according to Davison, Freifelder and Holloway (1964) by alkali treatment with the addition of formaldehyde (final concentration 2 per cent).

2.3. Hydrodynamic properties of the DNA molecules

The viscosity $[\eta]$ of the DNA molecules was determined in a rotation viscosimeter according to Zimm and Crothers (1962), and the sedimentation coefficients in an analytical ultracentrifuge (Spinco E, Beckman) at 260 m μ . Centrifuging was carried out at 20°C and at 15 220 r.p.m. Five different concentrations of one DNA sample were investigated in 0.165 M NaCl in the range between 5 and 30 μ g/ml. For calculation of the sedimentation coefficients at concentration c , the position of the middle of the boundary (50 per cent of the concentration, see figure 1 (b)) was used; this results in $S_{20, w, 0.5}^c$. The sedimentation coefficients obtained at different concentrations are extrapolated to zero concentration ($c=0$); this results in $S_{20, w, 0.5}^0$. From S^0 and $[\eta]$ it is then possible to determine the average molecular weight according to Mandelkern, Krigbaum, Scheraga and Flory (1952). Details of these procedures have been described earlier (Coquerelle *et al.* 1969).

2.4. Molecular weight distribution of the DNA molecules

The distribution of sizes of the DNA fractions obtained after irradiation was determined as follows. A step-wise analysis of the boundary was used to assess the distribution of the sedimentation coefficients S_1^0 at different initial concentrations c , and the individual distribution were extrapolated to the initial concentration of zero. According to Baldwin (1954), this eliminates the 'Johnston

Ogston effect' and the boundary sharpening effect. It results in a distribution of the S_i^0 values with different relative concentrations c_i at the boundary. The individual values of S_i^0 can be converted into the corresponding molecular weights M_i :

$$S_i^0 = K_s(M_i)^{a_s}, \quad (1)$$

where K_s and a_s are empirical constants. For native DNA we used $K_s=0.032$ and $a_s=0.405$; for denatured DNA, $K_s=0.055$ and $a_s=0.38$. Further details have been described elsewhere (Coquerelle *et al.* 1969, Weinert and Hagen 1968).

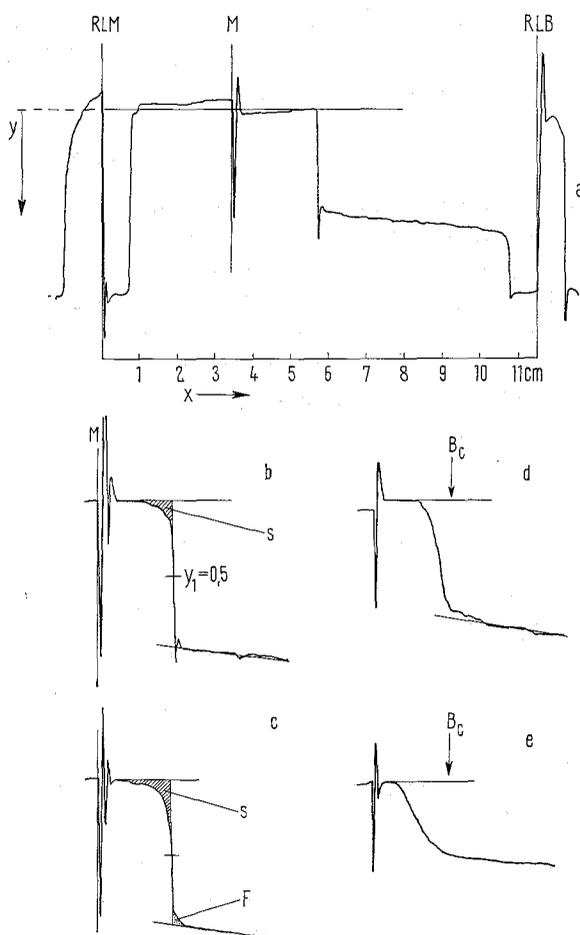


Figure 1. Densitometer trace of photographic image obtained by u.v.-absorption during sedimentation of T1 DNA, 15 220 r.p.m., 64 min after reaching the selected speed, except for (a) 72 min, (c) concentration of DNA solution. RLM, reference line meniscus; RLB, reference line bottom; $x \rightarrow$, direction of sedimentation; M, meniscus; $y_1=0.5$ position of the middle of the boundary (1b). (a) DNA of unirradiated T1, $c=31 \mu\text{g/ml}$. (b) 50 krad, $c=30.6 \mu\text{g/ml}$. The shaded field S represents the fraction of the slowly migrating, broken molecules. (c) 150 krad, $c=29.5 \mu\text{g/ml}$. The dotted field (F) represents the fraction of the DNA molecules, migrating faster than the intact DNA. (d) 500 krad, $c=20.3 \mu\text{g/ml}$; the arrow (B) shows the calculated position of the boundary of intact DNA molecules. (e) 1000 krad, $c=17.5 \mu\text{g/ml}$.

3. Results

3.1. Evaluation of the boundary

If DNA from unirradiated phages is centrifuged in the analytical centrifuge, a very sharp vertical gradient results, since all the molecules migrate at equal rates. In the densitometer (Analytrol, Beckman), this boundary turns out to be a vertical line (figure 1(a)). A corresponding investigation of the DNA of irradiated phages indicates after low doses (50krads) that some of the molecules migrate at a slower rate than the sharp boundary (figure 1(b), shaded field S). After a dose of 150krads, there will always be a fraction of molecules migrating faster than the uniform boundary (figure 1(c), dotted field F) as opposed to the more slowly-migrating fraction of broken molecules. After higher doses (500 and 1000krads), this fraction is no longer detectable; all the molecules migrate at a slower rate than the undamaged T1 DNA.

3.2. Average molecular weight and coiling properties of DNA

For quantitative determination of the degradation of DNA molecules, we calculated the average molecular weight $M_{s\eta}$ of the individual DNA samples. This results in an average value which corresponds approximately to the weight average mol. wt. M_w . The mean values of $M_{s\eta}$ obtained from the individual DNA samples from unirradiated and irradiated phages are compiled in table 1. For unirradiated T1 phages, an average molecular weight of 31×10^6 has been obtained, which is in agreement with the measurements by Bresler, Kiselev, Manjakov, Mosevitsky and Timkovsky (1967).

Dose (krads)	$S_{20,w}^0$	$[\eta]$ (dl./g)	$M_{s\eta} \times 10^{-6}$	$M_w \times 10^{-6}$	$M_n \times 10^{-6}$
0	35.13 ± 0.82	145.4 ± 6.5	31.27 ± 1.30	27.98 ± 0.83	27.98 ± 0.83
50	35.51 ± 1.89	144.8 ± 7.8	32.41 ± 3.77	29.28 ± 2.5	28.58 ± 2.47
100	35.58 ± 1.15	136.5 ± 11.0	31.81 ± 4.82	29.72 ± 0.0	28.45 ± 2.07
150	35.34 ± 2.99	138.1 ± 12.1	30.68 ± 5.21	28.23 ± 7.12	26.22 ± 5.71
250	34.57 ± 1.59	126.0 ± 2.5	28.34 ± 1.88	25.84 ± 1.31	22.38 ± 1.75
350	35.37 ± 1.04	124.0 ± 5.1	29.09 ± 1.21	25.31 ± 0.54	20.94 ± 1.92
500	30.59 ± 1.33	102.7 ± 4.9	21.13 ± 1.42	22.37 ± 0.39	17.31 ± 0.98
750	28.46 ± 1.87	83.2 ± 1.4	17.29 ± 1.87	18.87 ± 1.43	12.86 ± 0.97
1000	25.93 ± 0.06	65.5 ± 14.9	14.0 ± 0.63	20.80 ± 6.31	12.01 ± 0.36
350 before proteolysis	35.1 ± 1.9	113.75 ± 10.1	27.76 ± 4.01	26.02 ± 0.13	17.77 ± 1.09
350 After proteolysis	30.47 ± 1.41	96.2 ± 11.0	20.48 ± 2.59	20.82 ± 0.71	13.68 ± 0.54

Table 1. Properties of DNA from T1 phages irradiated in 0.1 M histidine.

The simultaneous determination of $S_{20,w}^0$ and $[\eta]$ allows the statement of whether the DNA preparations isolated from the phage exhibit the same hydrodynamic properties in solution as do undamaged DNA molecules. The values of $S_{20,w}^0$ and $[\eta]$ obtained with the individual preparations were plotted versus each other on a double logarithmic scale (figure 2). As has been described in

detail by Eigner and Doty (1965), the values of undamaged DNA molecules of different molecular weights are on a common curve, which is shown as a solid line in figure 2.

The DNA preparations from the unirradiated phages are largely on or near this curve. This applies also to those molecules which had been irradiated in the phage with low doses. After higher doses, especially after 350 krad, a number of DNA preparations are clearly on a second curve shifted parallel to the first one. In these preparations, the sedimentation coefficient is clearly larger in relation to viscosity, permitting the conclusion that there is a higher coiling density.

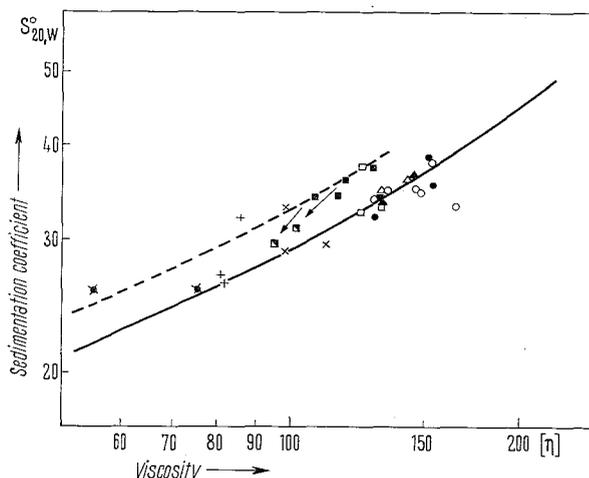


Figure 2. Relations between the sedimentation coefficient $S_{20,W}^0$, and the viscosity $[\eta]$ of DNA from irradiated T1. Each point represents an individual DNA preparation. Control \circ ; 50 krad \bullet ; 100 krad \triangle ; 150 krad \blacktriangle ; 250 krad \square ; 350 krad \blacksquare ; 350 krad after proteolysis \blacksquare ; 500 krad \times ; 750 krad $+$; 1000 krad \ast . The solid line is the calculated curve from Eigner and Doty (1965). For the dashed line and arrows, see text.

This higher coiling density of the DNA molecules from irradiated phages can be partly reversed by treatment with a proteolytic enzyme. After such treatment, the values of S^0 and $[\eta]$ approach the curve for undamaged DNA more closely and, at the same time, the molecular weight decreases slightly (see arrows in figure 2 and table 1). It should be emphasized that unirradiated DNA shows no change of S^0 or $[\eta]$ as a result of this treatment. Most probably, the DNA molecules from the irradiated phages exhibit a higher coiling density as a consequence of cross-links with the coat protein, where either branching with protein chains is the reason or the fixed protein prevents a complete expansion of the DNA molecule. Such cross-links with the coat protein are too infrequent after low radiation doses to change the coiling properties on the average. After high doses, breaks are so frequent that the DNA-protein cross-links can be detected only in a few DNA samples.

3.3. Breaks and cross-links in irradiated phages

As described in §2.4, an analysis of the boundary in the centrifuge cell results in the molecular-weight distribution and the weight average mol. wt M_w as well

as the number average mol. wt M_n . From the knowledge of both values, it is possible to calculate the number of breaks and cross-links (Coquerelle *et al.* 1969). In native DNA we define B_2 = frequency of double breaks per nucleotide pair, measured after a specific dose against a control, and C_2 = frequency of cross-links per nucleotide pair. Since the DNA was irradiated in the phage, it is not cross-links between different DNA molecules which are obtained but links between parts of the same molecule. The values of B_2 and C_2 calculated for the individual DNA samples are shown in figure 3.

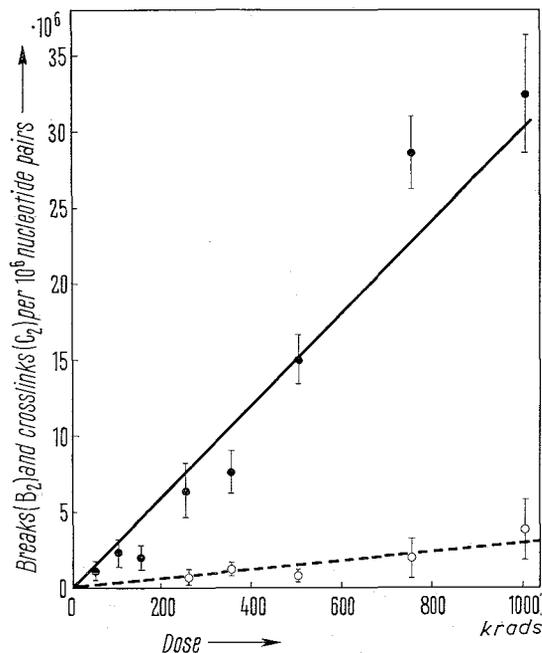


Figure 3. Formation of double-strand breaks and cross-links in the DNA of irradiated T1. B_2 , frequency of double breaks per nucleotide pair; C_2 , frequency of cross-links per nucleotide pair.

The frequency of breaks is about ten times higher than that of cross-links. The dose-effect curve for the double breaks in the irradiated phages could be represented in the dose-range investigated by a curve with an upward curvature; it appears however that from the measured data the emergence of a quadratic term is not statistically significant; we therefore assume a linear rise. The regression analysis then results in a probability for breaks of $p_2 = 3.0 \times 10^{-11}$ per nucleotide pair and rad. The frequency of cross-links C_2 is much lower; from the measured data a probability for cross-links results amounting to $p_2 = 0.3 \times 10^{-11}$ per nucleotide pair and rad. A proteolytic treatment does not influence the frequency of cross-links, hence, the intramolecular cross-links among the DNA sections (DNA-DNA-cross-links) can be distinguished clearly from the DNA-protein cross-links.

Moreover, some DNA samples were denatured after isolation from the phage and their molecular weight determined in the same way. From the decrease of M_n the frequency of breaks B_1 per nucleotide in the single strand is calculated,

as described earlier (Hagen 1967). From the increase in the frequency of breaks with dose the probability for breaks results as $p_1 = 37 \times 10^{-11}$ per nucleotide and rad.

Dose (krads)	$M_w \times 10^{-6}$	$M_n \times 10^{-6}$	$S_{20,w, 0.5}^0$	$[\eta]$ (dl./g)	$M_{s\eta} \times 10^{-6}$	$B_1 \times 10^5$
0	14.02	12.69	35.8	26	15.3	
25	12.66	10.36	35.1	27	15.15	0.55
50	9.49	5.57	24.85	31	9.14	3.12
100	7.52	4.68	23.3	26	8.05	4.17
150	7.08	4.38	20.4	16.5	5.02	4.63
220	4.16	2.65	16.78	8	2.73	9.22
250	4.9	3.06	19.6	25	6.07	7.7

Table 2. Properties of denatured DNA from T1-phages irradiated in 0.1 M histidine.

3.4. Breaks and cross-links in relation to phage inactivation

To correlate the single and double breaks and the cross-links with the inactivation of phages, we calculated, the fraction of phages in the DNA of which no radiation-induced lesions occurred. Considering single-strand breaks, the average number of breaks per phage genome is $B_1 \cdot M_0/m$, where M_0 is the mol. wt of the phage DNA (31×10^6) and m the average mol. wt of a nucleotide in T1-DNA

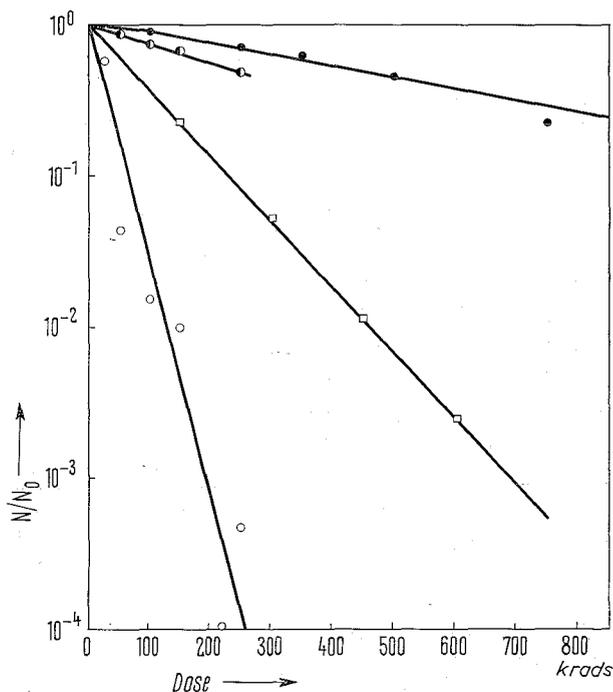


Figure 4. Inactivation of the plaque-forming ability and molecular alterations in DNA of irradiated T1. \square , fraction of biological active phages; \bullet , fraction of phages without double breaks or DNA-DNA-cross-links in their DNA; \circ , fraction of phages without single breaks in their DNA; \bullet , fraction of phages without double breaks or any cross-link, estimated by analysis of the uniform sedimenting boundary.

equalling about 308. The fraction of phages without radiation-induced single strand break (SSB) resulted in:

$$(N/N_0)_{\text{SSB}} = \exp - (B_1 M_0 / m). \quad (2)$$

In a similar way, the fraction of those phages can be calculated whose double strand (DS) contains neither double breaks nor DNA-DNA-cross-links:

$$(N/N_0)_{\text{DS}} = \exp - [(B_2 + C_2) M_0 / 2m]. \quad (3)$$

These fractions of phages without single breaks or with intact double strands are then compared with the fraction of surviving phages, the respective dose-effect curves being shown in figure 4.

For inactivation of the PFA a D_{37} of 95 krad results in agreement with earlier experiments in which the phages were irradiated in NB (Hotz and Zimmer 1963, Hotz 1966). The fraction of phages without single breaks exhibits a D_{37} of 27 krad. The fraction of phages with intact double-strand DNA resulted in a dose-effect curve with a small shoulder. From the mean slope a D_{37} of 500 krad results. All lesions on the double strand in the phage, including DNA-protein cross-links, are obtained by analysis of the uniform sedimenting boundary by the method of Freifelder (1965). However, this will lead to significant results only with low doses (cf. figure 1 (b), (c)). In this connection, we consider also the fraction which sediments faster than the undamaged DNA (F in figure 1 (c)). It is possible that this fraction F represents the DNA molecules cross-linked with the coat protein. On the whole, this method results in a D_{37} of 345 krad.

4. Discussion

It is possible by means of target theory to calculate from the D_{37} of the PFA of 95 krad the molecular weight of the target MG_T as 6.1×10^6 Dalton. Compared with the molecular weight of DNA of 31×10^6 as determined in the present experiments, this results in an inactivation probability of 0.20. In the DNA of the bacteriophage 305 eV is absorbed per inactivation, corresponding to a G-value of 0.33. A similar value is calculated from experiments by Freifelder (1965), conducted on T7 phages (table 3). In these and in the following calculations of the G-value we refer only to the mass of DNA and do not take into account the fact that some water is bound to the phage DNA also. According to Lauffer and Bendet (1954), a hydration of 50 per cent is probable. Moreover, North and Rich (1961) showed by x-ray-scattering investigations that DNA in the phage has a closely-packed specific arrangement which, otherwise, is found only in the highly-concentrated gel of less than 30 per cent water content. This specific arrangement of DNA in the phage is retained also when the phages are in an aqueous medium. If we take into account an average water-content of 40 per cent in the interior of the head of the phage, the G-values listed in table 3 must be multiplied by the factor of 0.6.

From the dose-effect curves for breaks and cross-links, the energy required for a single molecular event can be calculated. For the break of the single chain, the result is 86 eV, which corresponds to a G-value of 1.15. A similar value was obtained by Freifelder (1969) in B3 phages. Since the radicals formed in water also result in single breaks, the water bound in the head of the phage can be taken into account, leading to a G-value of 0.69. This is in good agreement with the G-values measured on DNA in mammalian cells and on dry DNA for the single break (table 3).

Condition of irradiation	Radiation induced lesion	Absorbed energy per lesion	G-value	Author
T1 phages in 0.1 M histidine T7 phages in 0.1 M histidine	Inactivation of PFA Inactivation of PFA	305† 365†	0.33 0.27	This publication Freifelder (1965)
DNA in T1 phages (0.1 M hist.) DNA in B3 phages (0.1 M hist.) Dry DNA in N ₂ DNA in <i>M. radiodurans</i> DNA in lymphoma cells	Single-strand break Single-strand break Single-strand break Single-strand break Single-strand break	86 100 140 140 150	1.15 1.0 0.716 0.71 0.66	This publication Freifelder (1969) Lücke-Huhle <i>et al.</i> (1970) Dean <i>et al.</i> (1966) Lett <i>et al.</i> (1967)
DNA in T1 phages (0.1 M hist.) Dry DNA <i>in vacuo</i> DNA gel (67 per cent H ₂ O) Dry DNA in N ₂	Double-strand break Double-strand break Double-strand break Double-strand break	1730 800 600 820	0.058 0.125 0.16 0.122	This publication Lett <i>et al.</i> (1961) Lett and Alexander (1961) Lücke-Huhle <i>et al.</i> (1970)
DNA in T1 phages (0.1 M hist.) Dry DNA <i>in vacuo</i> Dry DNA in N ₂ DNA gel (67 per cent H ₂ O)	DNA-DNA-cross-link DNA-DNA-cross-link DNA-DNA-cross-link DNA-DNA-cross-link	10700 1200 1260 1200	0.009 0.083 0.079 0.083	This publication Lett <i>et al.</i> (1961) Lücke-Huhle <i>et al.</i> (1970) Lett and Alexander (1961)
DNA in T1 phages (0.1 M hist.) DNA in T7 phages (0.1 M hist.)	Breaks and all cross-links (evaluation of the uniform boundary)	1100 732	0.090 0.137	This publication Freifelder (1968)

† Per 0.5×10^{-16} g.

Table 3. G-values for the inactivation of PFA, single-strand breaks, double-strand breaks and cross-links in phage DNA

For the double break we obtain a G-value of 0.058, which is less than has been measured by other authors on dry DNA and on DNA gels. Moreover, cross-links are much more infrequent than under comparable conditions in isolated DNA (0.009 relative to 0.083, table 3). Also, after irradiation of sperm heads a number of cross-links were found in their DNA, but hardly any breaks (Alexander and Stacey 1959), a result that caused these authors to suppose that bacteriophages were also inactivated mainly by cross-linking reactions. A possible reason for the low cross-linking frequencies demonstrated by us in phage may be the specific arrangement of the DNA molecule in the phage and its restricted mobility largely preventing the cross-linking of single DNA sections.

From changes in the coiling properties of DNA after irradiation of the phages and from the existence of a rapidly-sedimenting DNA fraction, it was possible to derive indications of a further molecular change in the DNA structure, i.e. cross-links between DNA and the coat protein. The frequency of these cross-links can be estimated by evaluation of the uniform sedimenting boundary, because in this way all events connected with the structure of double-stranded are taken into account. In this way a G-value of 0.090 is found. From the difference between this value and the G-value for double breaks plus that for intramolecular DNA-DNA-crosslinks, a G-value of some 0.02 results for the DNA-protein cross-links. This means that this type of cross-linking is not much more frequent than intramolecular cross-links within the DNA.

In summary: irradiation of T1 phage suspended in aqueous solution of 0.165 M NaCl+0.1 M histidine results in the average in 3.5 single breaks, 0.17 double breaks, 0.03 DNA cross-links and 0.06 DNA-protein cross-links per inactivation of the plaque forming ability of one phage particle.

Des bactériophages T₁ en suspension dans une solution d'histidine 0,1 molaire ont été irradiés aux rayons γ dans une source de ⁶⁰Co. Après irradiation, l'inactivation de la capacité de former des plages a été examinée et l'ADN a été extrait. Les altérations de structure ont été mises en évidence par les mesures de viscosité et de sédimentation. On a calculé le nombre de cassures dans l'ADN ainsi que les branchements à l'intérieur de la molécule d'ADN et les branchements entre ADN et protéine. On a montré que pour un phage inactivé, il y avait 3,5 cassures simples, 0,17 cassures doubles, 0,03 branchements dans la molécule d'ADN du phage et 0,06 branchements entre ADN et protéine.

Suspensionen von T1 Bakteriophagen in wässriger Lösung von 0,165 M NaCl+0,1 M Histidin wurden in einer ⁶⁰Co- γ -Quelle bestrahlt. Es wurde die Inaktivierung der Plaque bildenden Fähigkeit untersucht sowie die DNS aus den bestrahlten Phagen isoliert. Die Strukturveränderungen der DNS wurden durch Viskosimetrie und Sedimentationsanalyse bestimmt. Aus den Meßdaten kann die Zahl der Doppelstrangbrüche, der Einzelstrangbrüche sowie die der Vernetzungen innerhalb des DNS-Moleküls und der Vernetzungen zwischen DNS und Hüllprotein berechnet werden. Es ergab sich, daß pro inaktiviertem Phagen 3,5 Einzelstrangbrüche, 0,17 Doppelstrangbrüche, 0,03 DNS-DNS-Vernetzungen und 0,06 DNS-Protein-Vernetzungen eingetreten sind.

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