KERNFORSCHUNGSZENTRUM

KARLSRUHE

Mai 1968

KFK 796

Institut für Strahlenbiologie

The Action of Heat and Ionizing Radiation on the Infectivity of Isolated ϕX-174 DNA

G. Hotz, A. Müller

GESELLSCHAFT FUR KERNFORSCHUNG M. B. H.

KARLSRUHE

Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 60, No. 1, pp. 251–257. May, 1968.

THE ACTION OF HEAT AND IONIZING RADIATION ON THE INFECTIVITY OF ISOLATED φX-174 DNA

BY G. HOTZ AND A. MÜLLER

INSTITUTE OF RADIOBIOLOGY, NUCLEAR RESEARCH CENTER, KARLSRUHE, GERMANY

Communicated by Robert Sinsheimer, February 26, 1968

The biological activity of isolated single-stranded DNA of *E. coli* bacteriophage φX-174 can be assayed by the method of spheroplast infection developed by Guthrie and Sinsheimer.[1] Utilizing plaque formation as a convenient endpoint, Ginoza and Miller tested the effects of heat and ionizing radiation on such DNA.[2] In agreement with earlier work of Tessman[3] and Ginoza,[4] an inactivation efficiency per primary ionization of 1 was reported for X-irradiation of DNA in broth. In our experiments inactivation of dry φX-174 DNA by γ and electron irradiation was investigated between 4.2° and 433°K (160°C). On the basis of usual assumptions, an inactivation efficiency of at least 2 was measured at the highest temperature. This result and the observed temperature dependence of radiosensitivity require reconsideration of the basic mechanisms and concepts involved.

Materials and Methods.—Phenol-extracted φX-174 DNA was assayed by its ability to infect spheroplasts of *Escherichia coli* K12 following the method of Guthrie and Sinsheimer.[1] For inactivation experiments, aliquots of 0.1 ml of 4% Difco nutrient broth containing 0.01 μg of DNA were pipetted into glass ampules of 15-mm diameter and dried by slow evaporation. Subsequently, the ampules were evacuated for about 24 hr and sealed. No difference in sensitivity against heat or radiation was found between samples evacuated at 10^{-6} and 10^{-2} torr. Heat inactivation was carried out by immersion of the ampules in an oil bath maintained at a fixed temperature and was discontinued by transfer into ice water. For irradiation between −196°C and +120°C, a Co^{60}-γ-source delivering 1.5 Mrad/hr was employed. Above 120°C the rate of inactivation by heat alone was no longer a minor fraction of the total inactivation in the Co^{60}-source. Therefore, fast electrons of 10 MeV energy originating from a linear accelerator were used for irradiations. An electron beam of less than 10% inhomogeneity within a circular cross section of 3 cm^2 was directed at a thin-walled aluminum vessel containing the sample mount, a thermometer, and an electric heating coil. In order to avoid a substantial rise of the sample temperature due to absorption of radiation energy, the total dose was restricted to about 1 Mrad. This was delivered within 2 sec at an electron-pulse frequency of 10 Hz and a corresponding dose fractionation of 50 krad/pulse. At 4.2°K samples were irradiated during submersion in liquid helium contained in a liquid nitrogen-cooled double Dewar flask. For irradiations under these conditions high-energy bremsstrahlung of sufficient penetration and intensity yielding a dose-rate of 0.1 Mrad/min at the sample position was generated from a tungsten target intercepting an electron beam of 12 MeV energy. Ferrous sulfate was used for dosimetry of the Co^{60}-source. This method was checked by photometry of Perspex HX supplied by ICI and calibrated for dosimetry by the UK Panel on Gamma and Electron Irradiation. For the dosimetry of electrons and bremsstrahlung, Perspex HX was used in addition to inactivation of dry T1 bacteriophage of known sensitivity. Both methods agreed within limits of error which are 10% for irradiations above 4.2°K and 30% at 4.2°K.

Results.—Typical inactivation curves obtained at various temperatures with ionizing radiation and heat alone are plotted in Figure 1 versus radiation dose and time, respectively. Experimental points are well fitted by simple exponential kinetics except the initial parts of heat inactivation. This behavior is

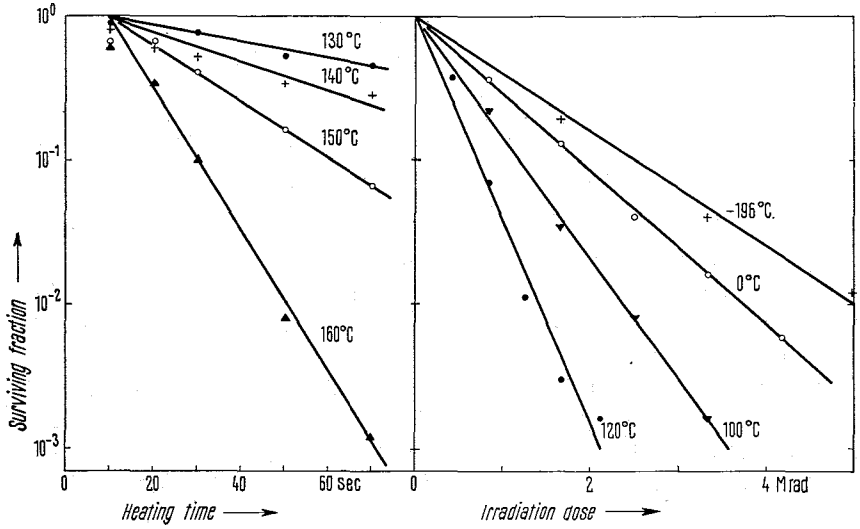

FIG. 1.—Heat and radiation survival curves of ϕX-174 DNA *in vacuo* dried from suspension in broth versus time and radiation dose, respectively. Radiation survival has been corrected for heat inactivation.

explained by the delay occurring between the immersion of the sample ampules into the oil bath at zero time and the actual attainment of the bath temperature by the sample material. Extrapolation to 100 per cent survival yields a time constant of about ten seconds for the initial temperature rise. Radiation survival has been corrected for heat inactivation.

The rate constants of exponential inactivation are equal to the reciprocals of 37 per cent doses and time, respectively, which are plotted versus temperature in Figure 2. It may be noted that heat inactivation is negligible below 120°C but increases rapidly above this temperature. Inactivation by irradiation is almost independent of temperature below 50°C but rises gradually at higher temperatures up to a sevenfold increase at 160°C.

An Arrhenius plot of the data shown in Figure 2 is presented in Figure 3. Rate constants of heat inactivation have been fitted by a straight line with a slope of 24.5 kcal/mol. Rate constants of inactivation by radiation are marked by full circles. The points above 4/1000°K have been connected by a straight line with a slope of 3.5 cal/mol which, however, is not significantly different from zero owing to an exceptionally large experimental uncertainty at liquid helium temperature ($1/T$ = 240/1000°K). The estimated error at this point is 30 per cent but only 10 per cent at all other temperatures. In any case, the slope of the straight line is too small to affect the extrapolated value below 4/1000°K, which is essentially constant in this relatively narrow range expanded in Figure 3. If the constant term is subtracted from the experimental points, the results are given by the open circles which have been connected by another straight line with a slope of 6.3 kcal/mol.

Inactivation by radiation has also been tested at −196°C, 0°C, and 130°C with

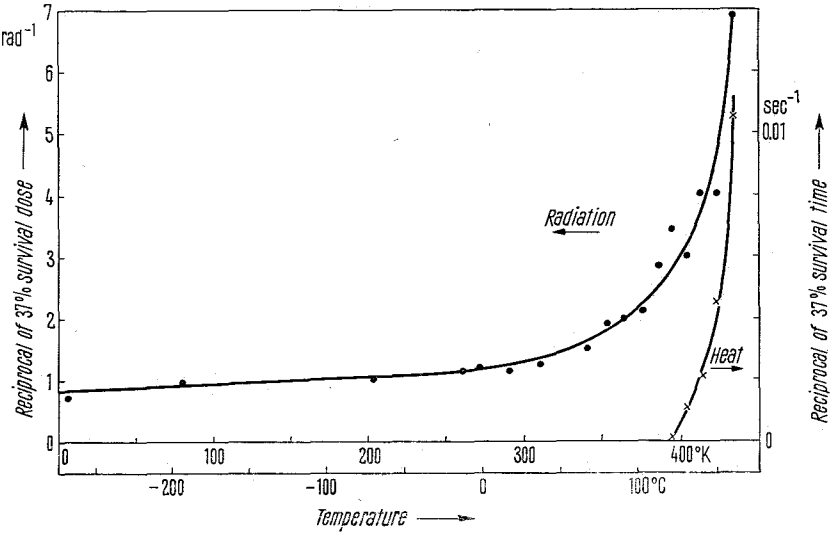

FIG. 2.—Reciprocal of 37% survival dose and time, respectively, versus temperature.

samples dried from broth supplemented by cystamine in 0.1 M concentration. Inactivation constants were reduced by factors of 1.6, 1.3, and 1.0, respectively. Thus the highest protective effect of cystamine is found at the lowest temperature, while it is entirely absent at 130°C.

Discussion.—The inactivation of dry ϕX-174 DNA by radiation is essentially independent of temperature below 50°C. In contrast to this result, the inactivation of dry whole-phage ϕX-174 has been found to depend on temperature in this region.[5, 6] In addition to a constant a second term with a slope of 1 kcal/mol was reported by Günther and Hermann in order to account for their experimental data.[6] A similar value was found for the inactivation of various proteins[7, 8] but is absent in our data on ϕX-174 DNA and may therefore be due to inactivation of the protein coat if whole phage are irradiated.

In order to visualize the variation of radiation energy W absorbed per inactivating event, this value is plotted on the right-hand side of Figure 4. W is calculated by using a molecular weight of 1.7×10^6 daltons.[9] The D_{37} which is proportional to W is also given. Below 30°C a mean value of 160 eV is found which is constant within the experimental limits of error but may decrease slightly with temperature rising. Above 30°C W drops in a linear fashion to 30 eV at 160°C.

The linear dependence on temperature above 30°C is represented by the equation: $D_{37} = c\,(T_D - T_o)$ where $c = 4.8$ krad/°C or $c = 0.012$ cal/gm°C and $T_D = 200$°C. This relation is identical to that derived theoretically by Norman and Spiegler.[10] The work of these authors is based on the assumption that the effects of densely ionizing radiations are essentially due to heating of the irradiated material in the core of traversing particle tracks. The thermal spike model, as it has been called, was applied to the results of protein inactivation in

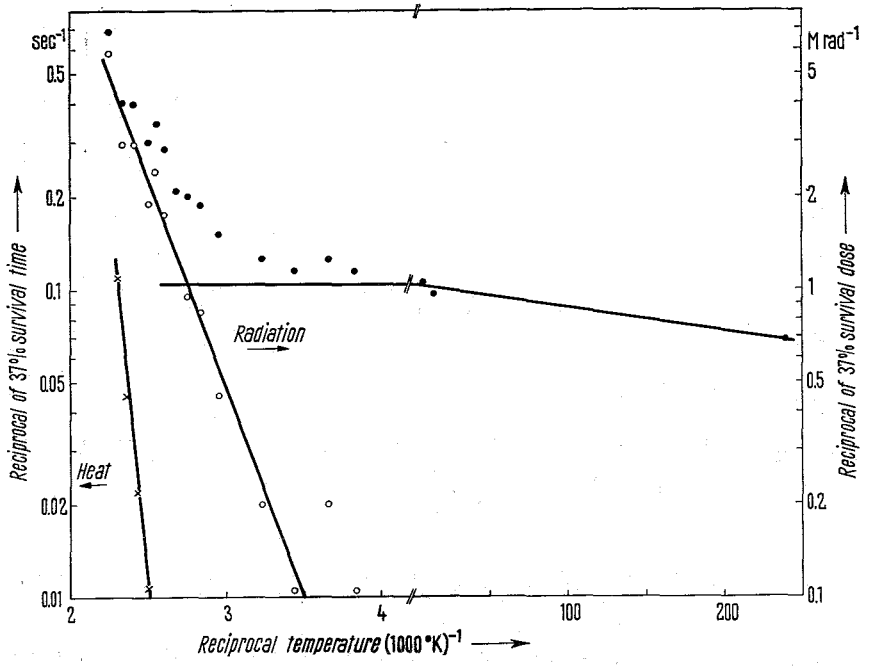

FIG. 3.—Reciprocal of 37% survival time and dose, respectively, versus reciprocal temperature (*full circles* and *crosses*). Open circles give the difference between full circles and the extrapolated straight line above 4/1000°K.

dependence on *LET* published by Brustad.[11] A good fit was obtained with $c = 0.3$ cal/gm°C and T_D between 225° and 325°C within an *LET* range between 30 and 10^4 MeV cm²/gm. The value of $T_D = 200$°C obtained from Figure 4 by extrapolation to zero ordinate is compatible with the decomposition temperature of Norman and Spiegler but c is about 30 times lower in our results. This means that the unlikely assumption of an extremely low heat capacity must be made in order to reach a temperature at which inactivation proceeds sufficiently rapidly. However, this is not the only objection which may be raised against the applicability of the thermal spike model, which its authors claimed to be valid only above 10^3 MeV cm²/gm. If the values of the inactivation constant below 30°C, which do not follow the same linear function as the points above this temperature, are subtracted from the total inactivation, the latter no longer lie on a straight line in the coordinates of Figure 4, but rather in the logarithmic scale of Figure 3. Therefore, the linear dependence of Figure 4 is regarded as fortuitous.

The thermal spike model was also applied to effects of sparsely ionizing radiation.[12] According to these calculations, the generation of hydrogen from irradiated toluene is caused predominantly by thermal decomposition in the tracks of δ-rays. Although such processes may occur in homogeneous substances, inactivation of DNA under the conditions of our experiments by such a mechanism can be excluded for the following reasons: Since 10^{-8} gm of DNA with an

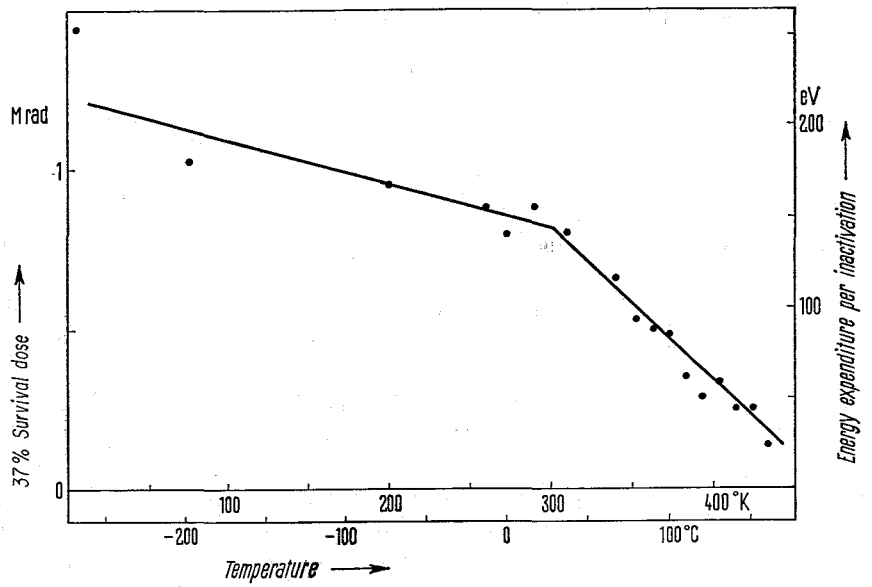

FIG. 4.—37% survival dose and energy expenditure per inactivation versus temperature.

efficiency of spheroplast infection of 10^{-3} is distributed in about 4 mg of dried broth, each infective DNA molecule is surrounded by about 10^{-9} gm of organic material. At unit density this is equivalent to a mean distance of about 10^{-3} cm. In comparison, $3 \cdot 10^{-7}$ cm was computed for the radius of a sphere of liquid toluene heated to 500°C above ambient temperature after absorption of 1 keV of energy.[12] Outside a sphere of 10^{-6} cm radius, the corresponding rise of temperature is already insignificant owing to the dependence on r^{-3}. For the range of a δ-ray of 1 keV, only 10^{-7} cm is given.[12] Since 10^{-6} cm is about 1000 times smaller than the mean distance between infective units, δ-rays cannot have an efficiency above unity and thus do not reduce the energy expenditure per inactivation below the mean value measured by physical methods.[13] This conclusion does not depend critically on the assumed homogeneous distribution of DNA, since clusters of up to 10^3 times the weight of one molecule of ϕX-DNA would still contain only one infective unit on the average.

The value of 24.5 kcal/mol found for pure heat inactivation (Fig. 3) is not very different from the 35 kcal/mol reported for the heat inactivation of ϕX-DNA in aqueous suspension and is typical of ordinary chemical processes.[14] In contrast to this result, the activation energy of 6.3 kcal/mol obtained from the Arrhenius plot of the combined actions of heat and radiation is much lower and rather typical of radical reactions. Inactivation by mobile radicals produced by irradiation not only would be compatible with the derived activation energy but also might explain the low value of $W = 30$ eV found at 160°C. If this value is compared to the mean energy per primary absorption event which has been reported[13] to lie between 60 and 120 eV, an inactivation efficiency of at least 2 is obtained for ϕX-174 DNA under these conditions.

An inactivation efficiency above 1 can only be understood if the energy absorbed in the surrounding medium contributes to the observed effect. The notion that this energy may simply be heat has been dismissed already. Substantial energy-transfer by small radicals is usually thought to be restricted to aqueous or other liquid systems and is defined as an indirect effect.[15] It may be reasoned that some water remained in our samples after drying and evacuating. In order to investigate this point, several samples were heated to 120°C for two hours prior to irradiation. Since no resulting modification of the radiosensitivities at 110° and 120°C ensued, it is concluded that water does not contribute significantly to the observed inactivation at high temperatures. Just as heat before irradiation did not modify the observed damage, heating after irradiation was equally ineffective.

Direct identification of radiation-produced atomic hydrogen in dry-phage DNA was recently achieved by Müller and Dertinger using ESR spectroscopy.[16] This result is complemented by an experiment of Jung and Kürzinger who found mobile radiation products capable of inactivating ϕX-174 DNA.[17] The experiments support the hypothesis that the temperature-dependent part of radiation damage is caused by atomic hydrogen. This hypothesis permits explanation of some results which so far have either not been explained at all or have been attributed to a contribution of δ-rays to inactivation in the case of densely ionizing radiation. Similarly, inactivation cross sections far in excess of geometrical cross sections which have been reported by Brustad[11] could well be due to the action of atomic hydrogen.

It is well known that separation and addition reactions of atomic hydrogen are not restricted to DNA, but occur ubiquitously in all kinds of organic material containing hydrogen. Therefore, at least part of the atomic hydrogen reacting with DNA originates from the surrounding dehydrated broth in our experiments, since the DNA constitutes an extremely small fraction of the total sample weight. If the composition of the bulk material is changed, the reactions of atomic hydrogen with DNA may be affected as well. In order to test this contention, we have performed several experiments. However, too many important parameters which may influence the results are as yet unknown, e.g. the thermal and radiative decomposition reactions above 100°C. For these reasons, the results cannot be taken to prove any particular mechanism. Nevertheless, it is demonstrated that the radiosensitivity of DNA may be enhanced not only by heat but also by changing the material surroundings. Thus, the inactivation was increased about twofold if broth was replaced by NCE-buffer solution or if the suspension in broth was dialyzed for 24 hours against distilled water. The addition of cystamine to the broth had no effect at high temperatures but only at room temperature and below.

Summary.—The sensitivity of isolated dry ϕX-174 DNA against inactivation by heat and ionizing radiation between 4.2 and 433°K (160°C) was investigated by assaying the plaque formation after infection of *E. coli* spheroplasts. The effect of heat alone was found to rise steeply above 120°C, while the radiosensitivity begins to increase markedly at 50°C but remains practically constant at lower temperatures. Both actions were observed up to 160°C, at which tem-

perature the radiosensitivity was about seven times that at room temperature, corresponding to an inactivation efficiency of at least 2. This high value is attributed to the participation of radiation-produced atomic hydrogen in the total inactivation. This conclusion is supported by an activation energy of 6.3 kcal/mol by which the temperature-dependent inactivation is characterized. The corresponding value for heat inactivation is 24.5 kcal/mol. The results are also discussed in terms of the thermal spike model. It is concluded that the partial agreement is fortuitous.

The authors gratefully acknowledge the capable technical assistance of Miss R. Mauser. We also thank Mr. Rudolf for performing the electron irradiations at the Institute of Food Technology and Mr. Uenzelmann for performing the irradiations at liquid-helium temperature.

[1] Guthrie, G. D., and R. L. Sinsheimer, *J. Mol. Biol.*, **2**, 297 (1960).
[2] Ginoza, W., and R. C. Miller, these PROCEEDINGS, **54**, 551 (1965).
[3] Tessman, I., *Virology*, **7**, 263 (1959).
[4] Ginoza, W., *Nature*, **199**, 453 (1963).
[5] Hotz, G., *Chemische und physikalische Beeinflussung der Strahlenwirkung auf Coliphagen* (TH Karlsruhe: Habilitationsschrift, 1965).
[6] Günther, H. H., and K. O. Hermann, *Z. Naturforsch.*, **22b**, 53 (1967).
[7] Henriksen, T., *Radiat. Res.*, **27**, 694 (1966).
[8] Günther, W., and H. Jung, *Z. Naturforsch.*, **22b**, 313 (1967).
[9] Sinsheimer, R. L., *J. Mol. Biol.*, **1**, 43 (1959).
[10] Norman, A., and P. Spiegler, *J. Appl. Phys.*, **33**, 2658 (1962).
[11] Brustad, T., *Radiat. Res.*, **15**, 139 (1961).
[12] Ingalls, R. B., P. Spiegler, and A. Norman, *J. Chem. Phys.*, **41**, 837 (1964).
[13] Rauth, A. M., and F. Hutchinson, *Biological Effects of Ionizing Radiation at the Molecular Level* (Vienna: International Atomic Energy Agency, 1962), p. 25.
[14] Fiers, W., and R. L. Sinsheimer, *J. Mol. Biol.*, **5**, 420 (1962).
[15] Zimmer, K. G., *Studies on Quantitative Radiation Biology* (Edinburgh: Oliver and Boyd, 1961).
[16] Müller, A., and H. Dertinger, *Z. Naturforsch.*, **23b**, 83 (1968).
[17] Jung, H., and K. Kürzinger, in preparation.