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Temperature dependence of radiation sensitivity in the dry state: A model derived from experiments using atomic hydrogen

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The effect of temperature on the inactivation rate of phage T1 *in vacuo* by radiation-induced diffusible radicals has been studied. Reactive species were liberated by 2-MeV protons impinging on thin foils of organic material and permitted to reach phage samples arranged at some distance. Thus, the sites of radical production and radical reaction were separated in space. Two different modes of exposure were used: the temperature either of phage samples only (method A), or both of foil and samples (method B), could be varied from 100°K to 340°K. Activation energies of $0{\cdot}65 \pm 0{\cdot}1$ kcal/mole and $0{\cdot}95 \pm 0{\cdot}1$ kcal/mole, respectively, were derived from the temperature dependence of damage. The latter value, simulating inactivation by secondary processes in ionizing radiation, strongly supports the hypothesis that the activation energy of 1 kcal/mole, frequently reported in the literature for radiation damage, is related to H radical action. Using the results of both experiments, an interpretation of the temperature effect is given by separating the overall activation energy into components of 0·3 kcal/mole necessary for H atoms to overcome ' cage-effects at the parent-molecule ' and of 0·65 kcal/mole to maintain diffusion by freeing the radicals from trapping sites.

1. Introduction

Since the early days of radiation biology, it has been hoped that the temperature dependence of radiation injury—among other experimental parameters—would give a clue to our understanding of the mechanisms leading to observable radiation damage. After it was found that the effect of ionizing radiation was decreased at low temperatures in the dry state, systematic investigations were made of the variation of the radiation sensitivity of enzymes (Setlow 1952, 1955, Pollard, Powell and Reaume 1952, Setlow and Doyle 1953), phage (Adams and Pollard 1952, Bachofer, Ehret, Mayer and Powers 1953, Uenzelmann 1968), bacterial spores (Webb, Ehret and Powers 1958, Powers, Webb and Ehret 1959), and most recently of infectious DNA (Hotz and Müller 1968), over a wide range of temperature. Irradiations were carried out with almost the complete spectrum of ionizing radiations, ranging from γ-rays to heavy ions. The investigations were extended from the temperature of liquid helium (Brustad 1964, Hotz and Müller 1968, Uenzelmann 1968) to the limit set by thermal inactivation (Pollard *et al.* 1952, Powers *et al.* 1959, Brustad 1964, Fluke 1966, Hotz and Müller 1968, Kürzinger and Jung 1968 a). When it was found that under certain conditions the inactivation cross-section surpasses the geometric dimension of a molecule, several hypotheses concerning excitations or ' some indirect effect ' from surrounding molecules were discussed to account for this astonishing fact. Augenstein (1963) postulated energy and charge migration, and Norman and Spiegler (1962) suggested the ' thermal-spike '-model.

2.3. *Exposure chamber*

An apparatus was constructed for the exposure of 50 samples in one run using two different modes of exposure. The principles of these two methods are shown schematically in figure 1. With method A the samples could be cooled or heated to the required temperature, whereas the emitting foil was kept at room temperature for all measurements. Thus, a constant yield of diffusible agents was obtained and the reaction rate of H atoms could be investigated with samples at various temperatures. With method B, both foil and samples were brought to the chosen temperature, thus imitating closely the conditions in direct irradiation of macromolecular material, where the entity supplying the mobile radical and the molecule damaged by the latter are both at the same temperature.

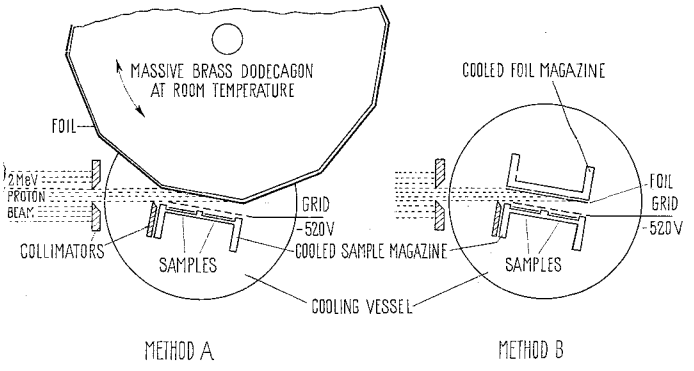

Figure 1. Schematic representation of the arrangements for exposing specimens to atomic hydrogen generated by bombarding a foil of organic material with 2 MeV protons according to methods A and B (see text).

The geometric conditions were identical in both methods. The collimated proton beam hit the foil at a small angle (9°), and the sample discs were positioned opposite to the foil but shielded from the incident particles. A grid, maintained at a negative potential of 520 V, was interspersed between the foil and the discs to screen off secondary electrons emitted by the foil. The probability of a proton leaving the foil again by the front surface was less than 10^{-7}, as estimated from multiple scattering theory. A schematic view of the exposure chamber is given in figure 2. Instead of the usual assembly of a rotating irradiation plate, a linear magazine-like arrangement was chosen so that the two methods which have been described could be used with the same apparatus. Guide tubes of rectangular profile are inserted into a barrel-like vessel that can be filled, for example, with cooling liquid. In the middle of this cooling vessel where the irradiation of the foil and the exposure of the samples take place, a slot is cut, wide enough to allow the exposure of one pair of samples. A solid brass dodecagon, to which the foil is attached (method A), can be lowered into this irradiation slot, fitting closely but without direct contact. The wheel disc is turned by means of a crank and can be fixed exactly at angular intervals of 30°. Two magazines, one for the samples and one for the foil (method B), can be slid into the guide tubes. The sample magazine provides for 50 glass discs

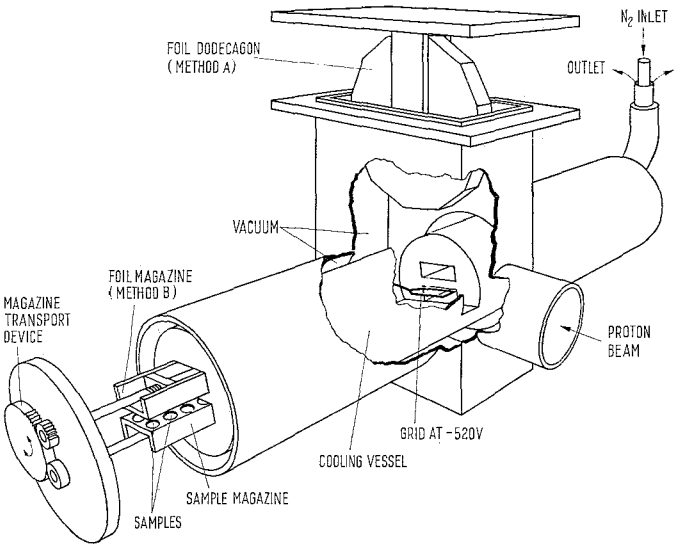

Figure 2. Schematic view of the exposure chamber. The arrangements for both modes of exposure (methods A and B) are shown in one drawing.

fitting into shallow holes. For experiments according to method B (foil and samples at same temperature) the brass disc is removed and the foil fastened instead on to the upper magazine, so that the foil is in exactly the same position as in method A. Suitable beam collimators ensure that in both methods identical foil areas are irradiated. The lengths of the magazines are adjusted, so that they can be sheathed completely by each of the two barrel halves. By this extensive sheathing optimal temperature adjustment between the thermostat vessel and the magazines is achieved. In constructing the foil wheel, special care was taken to provide good thermal contact with the cover sheet to which it was fastened by solid supports. The magazines can be moved to and fro across the irradiation slot by means of gear-driven screw rods suspended in vacuum tight shaft bearings. The exact positions for exposure are indicated by a micro-switch; the number of the respective pair of samples is also shown. During operation the chamber was evacuated to a pressure of $1\text{–}3 \times 10^{-5}$ torr.

2.4. *Temperature adjustment and measurement*

The thermostat vessel can be filled with cooling liquid or gas through the inner of two coaxial pipes. Reflux is maintained through the outer pipe. In most cases cold gaseous nitrogen was used. By controlling the gas flow the desired sample temperature could be stabilized. For the experiments at elevated temperatures the thermo-barrel was flushed with hot air. The lowest temperatures (about 100°K) were obtained by filling the cooling vessel with liquid nitrogen. For temperature measurement in one of the guide tubes, a thermocouple was installed in such a way that it was not in contact either with the tube wall or with the inserted magazine. Comparative measurements carried out with a couple fixed to the magazine in place of a sample disc indicated that in no case did the indicated values differ by more than 2°. Similarly, it could be shown that cooled samples when brought into the exposure slot maintained

their temperature for some time, even if the foil just opposite was at room temperature (method A). Only after more than 30 sec was a slow temperature rise observed, owing to photon radiation from the foil. If, however, the thermocouple was fastened to the foil wheel placed in the slot of the cooled barrel, the temperature of the solid metal disc as well as of the foil fell only a few degrees below room temperature when hot air was blown over the cover.

2.5. *Foils*

A 6 μ-thick foil of polyethyleneterephthalate (trade mark: Hostaphan) was used in method A. In method B the foil should assume the temperature of the particular experiment and not be warmed perceptibly by the incident proton beam. Therefore, an extremely thin coating, adhering tightly to the metal, was required, so that foil and support were an integral part and the absorbed energy could disperse to the total mass of the magazine. Polyvinylformal (trade mark: Formvar) was therefore dissolved in chloroform to a concentration of 1–5 mg/ml. and the magazine immersed repeatedly into this solution, each time allowing the solvent to evaporate. Foils of the desired thickness and optimal adhesion could thus be obtained. The inhomogeneities of the coating amounted to ± 20 per cent. For the serial experiments according to method B a foil thickness of 1700 Å was chosen.

2.6. *Irradiation and exposure procedure*

The exposure chamber was installled at the muzzle of a Van de Graaff accelerator (High Voltage Eng). A thin foil of nickel defocused the 2 MeV proton beam so that the intensity of the beam was uniform across the aperture of the diaphragm. In the serial experiments the particle flux was $1{\cdot}56 \times 10^{12}$ p/cm^2 sec corresponding to a beam current of $2{\cdot}5 \times 10^{-7}$ A/cm^2. In each run 50 samples could be exposed to radical attack. An additional dozen control samples were subjected to identical conditions of pressure and temperature. After loading, the chamber was evacuated for several hours to remove any traces of gas or moisture from the samples. The irradiation was started when the temperature chosen for the particular run was stabilized. If the exposure time was longer than 10 sec, the irradiation was carried out in fractions: every 10 sec the pair of samples and the irradiated foil section were exchanged. Then, during all phases of an experiment, temperature conditions remained constant within the foil, which in method B was heated by irradiation. This is necessary to obtain constant radical yield. Moreover, the temperature of the samples (see above) is well defined in this case and wearing out of the foil is avoided. An absolute measurement of the exposure yield was not made. The exposure time, i.e. the sum of the single fractions of irradiation, was taken as a relative measure for the delivered ' radical-exposure '.

3. Results and discussion

In all the experiments the survival rate, i.e. the ratio of the plaque counts found after exposure to that of non-exposed control samples, decreases continually with increasing exposure time and approaches an asymptotic value. A typical inactivation curve, obtained with phage T1 using method B at room temperature, is given in figure 3. It is not possible to inactivate the samples below some value between 5 and 30 per cent depending on sample preparation.

This value was determined for each experiment by long exposures providing good statistics. As deduced from irradiation of infectious phage-DNA with slow protons (Kürzinger and Jung 1968 b), part of the test material is enclosed in minute crystals of buffer substance. This part cannot be reached by hydrogen atoms because of their short penetration depth and gives rise to the remaining indestructible fraction of biological activity. A constant fraction, consequently, has to be subtracted from the experimental data. In a semi-log plot, the corrected points fall satisfactorily on a straight line (figure 3). The exposure time necessary for inactivation to a survival rate of 37 per cent (t_{37}), as obtained

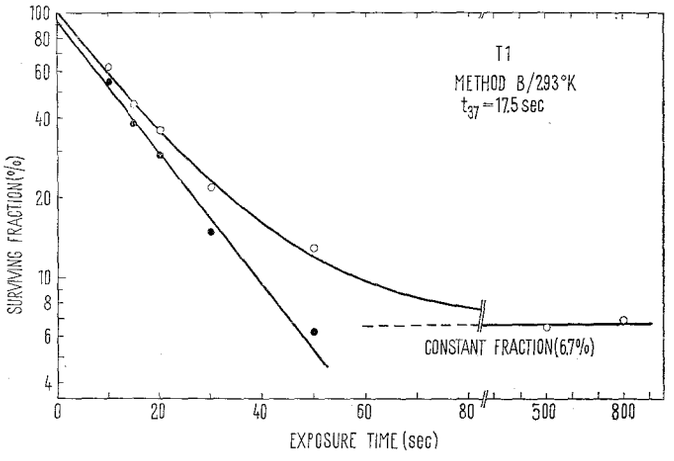

Figure 3. Typical inactivation curve obtained after exposure of dry phage T1 to radiation-induced atomic hydrogen.

from the slope of this line, can be taken as a measure of the resistance against H radicals. Vice versa, the reciprocal value ($1/t_{37}$) provides a relative measure for the reaction cross-section of a molecule, i.e. for the sensitivity against the inactivating radicals. The inactivation rate, of course, depends on the number of H atoms liberated per unit time and area and, thereby, on the intensity of the primary proton beam. This dependence is shown in figure 4 as found in a series of experiments with method B. The reciprocal of 37 per cent exposure time ($1/t_{37}$) is plotted versus proton beam current on a double-log scale. In this plot as well as in all the following ones showing values of $1/t_{37}$ each experimental point is derived from a complete inactivation curve of the type shown in figure 3. The straight line drawn through these points is described by the equation:

$$1/t_{37} = c \,.\, i^{0{\cdot}82} \ (\mathrm{sec}^{-1}), \qquad (6)$$

with $c = 20\,000$ A^{-1} sec^{-1} cm^2 if the proton flux is given in $\mathrm{A/cm}^2$ (c is a constant connected with the experimental set-up and depending on type and energy of the radiation releasing the radicals). From equation (6) the existence of a dose-rate effect can be seen clearly (exponent $\neq 1$): the damage to the samples does not increase linearly with the intensity of the proton beam hitting the foil. As the ' radical-sensitivity ' of the test material cannot have altered there is only one reasonable explanation for this effect: the recombination rate of the H atoms liberated depends on concentration. Because of the higher radical concentration at high proton current, relatively more H atoms recombine to hydrogen gas and

fewer are available for the formation of macromolecular radicals by addition and/or abstraction. All the experiments cited subsequently were carried out at a uniform beam current of $2{\cdot}5 \times 10^{-7}$ A/cm^2.

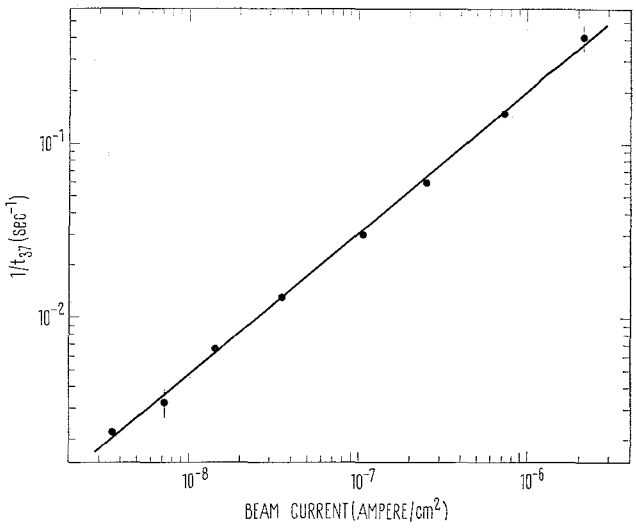

Figure 4. Reciprocal of 37 per cent survival exposure time for the inactivation of dry phage T1 by radiation-induced atomic hydrogen relative to the intensity of the primary 2 MeV proton beam (method B, room temperature).

It was further necessary to investigate the influence of foil material and thickness on the radical yield and, thereby, on the inactivation rate of the samples to provide a correlation between the experiments with 6 μ foil of Hostaphan according to method A and the studies by method B done with a Formvar film of 1700 Å. For that purpose, the foil magazine (method B), in turn, was coated with layers of either material and varying in thickness. As seen from figure 5, neither the type of material (for the two cases studied) nor

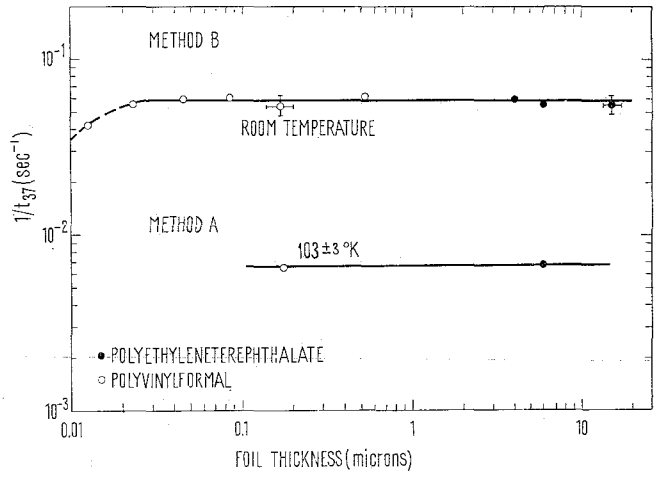

Figure 5. Dependence of reciprocal of 37 per cent survival exposure time for the inactivation of dry phage T1 by radiation-induced atomic hydrogen on material and thickness of the irradiated foil.

the thickness of the foil influences the radical yield. The decrease of the $1/t_{37}$-value for the thinnest layer used is subject to some uncertainty. Probably, in this case, the inhomogeneity of foil thickness exceeds 20 per cent, the value applying for the other experiments. It was not possible to obtain any reliable control data for foil thickness zero, i.e. for irradiation of the bare magazine, as it is virtually impossible to free a metal surface from minute traces of adsorbed substances containing hydrogen.

Some of the inactivation curves as determined for various temperatures by method A and method B, respectively, are given in figures 6 and 7. In both exposures a very pronounced temperature effect is found: within the temperature

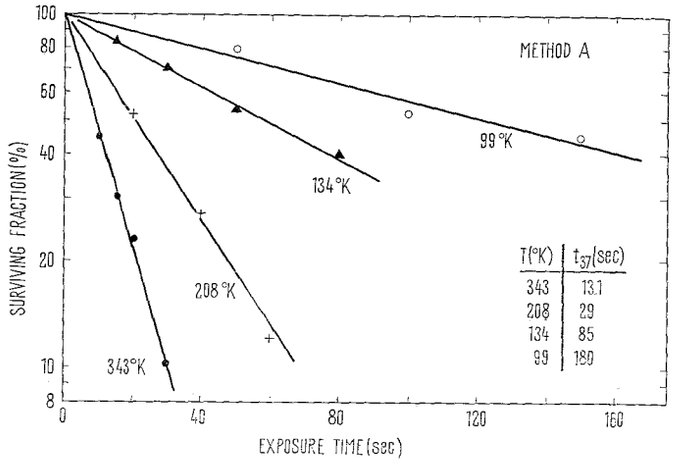

Figure 6. Inactivation of dry phage T1 by exposure to radiation-induced atomic hydrogen (specimens at various temperatures).

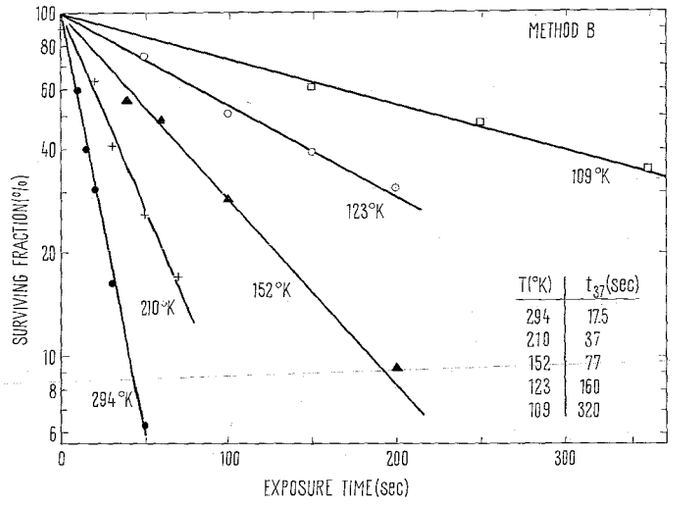

Figure 7. Inactivation of dry phage T1 by exposure to radiation-induced atomic hydrogen (specimens *and* irradiated foil at various temperatures).

range investigated the rates of inactivation by H atoms vary by more than one order of magnitude. The highest temperature at which we could obtain a result was 343°K (=70°C). In this case, thermal inactivation (measured in the control samples) already amounted to 85 per cent whereby the statistical error of plaque counts was increased considerably. At still higher temperatures, the control samples were inactivated almost completely.

The summary of all the experiments performed with the two methods is given in figure 8. The graph represents the usual Arrhenius plot where the sensitivity or reaction cross-section $1/t_{37}$ is plotted versus the reciprocal of

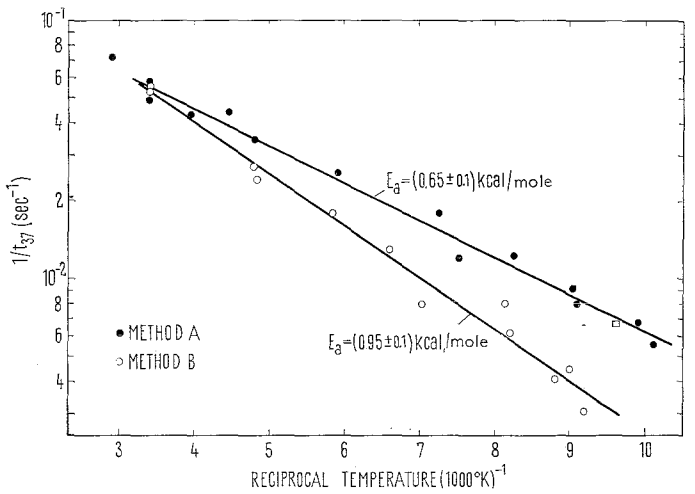

Figure 8. Reciprocal of 37 per cent survival exposure time for the inactivation of dry phage T1 by radiation-induced atomic hydrogen versus reciprocal of temperature (Arrhenius plot). Open circles: 1700 Å Formvar foil, method B. Closed circles: 6 μ Hostaphan foil, method A. Open square: 1700 Å Formvar foil, method A.

temperature. By this method, as seen from equation (1), the slope of a straight line can be used to calculate an apparent activation energy for the process investigated. For either procedure of exposure, the experimental points can be fitted by a straight line. These curves are given by the equations:

$$1/t_{37} = c_A \cdot \exp(-E_a^A/RT)\ (\text{sec}^{-1}) \quad \text{(for method A)} \tag{7}$$

and

$$1/t_{37} = c_B \cdot \exp(-E_a^B/RT)\ (\text{sec}^{-1}) \quad \text{(for method B)}, \tag{8}$$

with $c_A = 0{\cdot}164$ sec^{-1} and $c_B = 0{\cdot}258$ sec^{-1}. The activation energies evaluated for the two methods amount to $E_a^A = 0{\cdot}65$ kcal/mole and $E_a^B = 0{\cdot}95$ kcal/mole, respectively. Within the investigated range of temperature there is no indication of a constant term independent of temperature, quite at variance with observations made in experiments using radiations: in these studies a temperature-independent contribution with $E_0 = 0$ and another one with $E_1 = 1$ kcal/mole were nearly always found for a broad variety of radiation types and objects investigated. In some radiation experiments extended to elevated temperatures a third component $E_2 = 3$ to $6{\cdot}5$ kcal/mole was observed, though E_2 is calculated by double subtraction leading to considerable experimental uncertainty.

The activation energy $E_a^B = 0{\cdot}95 \pm 0{\cdot}1$ kcal/mole, derived for method B where conditions are in a way similar to radiation inactivation (foil and samples at same temperature) is, within experimental error, the same as the temperature exponent $E_1 = 1$ kcal/mole from equation (1). This agreement lends considerable support to the hypothesis that the temperature dependence of radiation inactivation in dry state is due to the lesions contributed to the total radiation injury by atomic hydrogen. So far an absolute measurement of the radical output could not be performed. There is, however, a way of calculating at least the order of magnitude of the measured effect: one can compare the 2-MeV proton ' dose ' necessary for a certain inactivation (e.g. D_{37}) by direct irradiation of phage samples (Hermann 1966) with the proton ' dose ' needed for comparable inactivation by indirect irradiation of the foil in this experiment. For that purpose, one has to take into account the geometrical conditions of the exposure chamber (only radicals entering in a solid angle of $4\pi/7$, on the average, have a chance of hitting the samples) and the magnitude of the temperature dependent fraction of the inactivation rate in Hermann's study (40 per cent). It turns out that the rate of inactivation by diffusible radicals in the samples completely separated from the irradiated foil amounts to 12·5 per cent of the inactivation rate resulting from the attack of secondary products in the phage samples in direct irradiation. This statement makes the implicit assumption that the output of diffusible radicals is similar in phage samples and organic foil, which is not unreasonable. This is a rather crude calculation, but one can be quite sure that the order of magnitude is correct. As the radiation-induced radicals will react preferably with molecules right next to their origin, the percentage of 12·5 (found in samples completely separated from the sites of radical induction) seems to be high enough to illustrate that the measured radical reaction is not a minor component, but may account very well for the temperature dependence of radiation damage, as outlined above. To find out how far the component E_2 with 3–6·5 kcal/mole also originates from radical action, investigations will be extended to higher temperatures, as is possible for objects showing high thermal resistance (e.g. RNase, ΦX 174–DNA).

If, for the experiments using method B (H radical supply and samples at equal temperature conditions) an activation energy of about 1 kcal/mole is found, what conclusions can be drawn from the value of $E_a^A = 0{\cdot}65$ kcal/mole obtained with method A? Here the only difference was that the compartment providing the H atoms was at constant temperature during all the experiments, i.e. the radical yield remained constant. The temperature dependence of the liberation of atomic hydrogen must, therefore, account for the difference between the two experiments, i.e. the liberation of H atoms, obviously, needs an activation energy of 0·3 kcal/mole. The primary separation of H atoms as a consequence of the charge shifting caused by ionization will be independent of temperature, to a first approximation. The split-off H atoms, nevertheless, may be fixed to their original sites by means of steric restrictions, e.g. by side-chains of the ' parent-molecule '. This cage effect postulated by Franck and Rabinowitsch (1934) would lead to an increased rate of recombination with the macroradical (cf. equation (2)). The difference between the temperature exponents $E_a^B - E_a^A = 0{\cdot}3$ kcal/mole is probably the activation energy required to overcome cage effects and to liberate split-off H atoms from their captivity. This amount of energy, therefore, will be symbolized by E_a^L (L stands for ' liberation '). The influence of temperature on diffusion of H atoms within the foil is neglected in

this consideration, but it is intended to check this interpretation concerning E_a^L by varying the temperature of the foil while keeping the sample temperature constant. On the other hand, the value of $E_a^D = 0{\cdot}65$ kcal/mole found with method A corresponds to the temperature dependence of the rate of causing damage (D) alone. This energy, however, is not necessarily required only for direct reaction of H atoms with biomolecules (equations (4) and (5)), but also for the maintenance of hydrogen diffusion within the sample material. The diffusing H atoms can be captured in trapping sites, i.e. in shallow potential wells, existing abundantly in the complicated structures of organic compounds. If their energy is not sufficient to leave these traps, H radicals can only react by recombination with another diffusing H atom to render both harmless. By expenditure of the activation energy E_a^D (one has to be aware of the statistical energy distribution), radicals are removed from their trapping sites and, thereby, get a further chance to form abstraction and/or addition radicals with biomolecules. The sensitivity of phage T1 against radiation-induced H radicals, dependent on temperature, formulated in equation (8) now can be described in a more detailed form:

$$1/t_{37} = c_B \cdot \exp\left[-(E_a^L + E_a^D)/RT\right] (\text{sec}^{-1}), \tag{9}$$

with $E_a^L = 0{\cdot}3$ kcal/mole and $E_a^D = 0{\cdot}65$ kcal/mole.

The simplified interpretation given here of the influence of temperature on the rate of inactivation by ionizing radiation may be still hypothetical in detail. However, the demonstration achieved by the present investigation that the activation energy of 1 kcal/mole found in most radiation studies may be attributed to temperature-dependent radical reactions is hardly open to doubt.

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On a étudié l'influence de la température sur la taux d'inactivation du phage T1 (sous vide) par des radicaux diffusibles induits par irradiation. Les agents réactifs (atomes H) sont produits par bombardement (protons, 2 MeV) d'une mince feuille de matière organique, et diffusés dans l'espace pour atteindre les échantillons biologiques situés à quelque distance. Deux modalités d'étude ont été employées en variant soit la température des échantillons phages seulement (méthode A), soit à la fois la température de la feuille bombardée et celle du matériel biologique (méthode B). Les mesures ont été effectuées entre 100 et 340 °K. Les changements du taux d'inactivation des phages avec la température ont permis de déterminer une énergie d'activation de $0{,}65 \pm 0{,}1$ kcal/mole (méthode A) et de $0{\cdot}95 \pm 0{,}1$ kcal/mole (méthode B). La seconde valeur confirme l'hypothèse selon laquelle l'énergie d'activation de 1 kcal/mole généralement rapportée dans la littérature lors de l'inactivation par les radiations ionisantes doit être reliée à l'action des radicaux H. On interprète de la façon suivante les résultats obtenus selon les deux méthodes: l'énergie d'activation totale est constituée de deux composantes, l'une, de 0,3 kcal/mole, est nécessaire pour vaincre les empêchements stériques au sein de la molécule-mère, l'autre, de 0,65 kcal/mole, pour maintenir la diffusion des radicaux hors des centres de captage.

An T1-Phagen wurde die Temperaturabhängigkeit der Inaktivierungsrate (im Vakuum) durch strahleninduzierte diffusible Radikale untersucht. Reaktionsfähige Agentien wurden durch Beschuß mit 2 MeV-Protonen aus dünnen Kunststoffolien ausgelöst und konnten auf die räumlich getrennt angebrachten Proben treffen. In zwei unterschiedlichen Expositionsverfahren wurden entweder nur die Temperaturwerte der Proben allein (Methode A) oder der Proben und der bestrahlten Folie zusammen (Methode B) zwischen 100°K und 340°K variiert. Aus der Temperaturabhängigkeit der Schädigungsrate ließen sich Aktivierungsenergien von $0{,}65 \pm 0{,}1$ kcal/Mol bzw. $0{,}95 \pm 0{,}1$ kcal/Mol ableiten. Der zweite Wert, der den Verhältnissen bei direkter Bestrahlung entspricht, weist darauf hin, daß die für die Strahlenschädigung häufig gefundene Aktivierungsenergie von 1 kcal/Mol mit H-Radikal-Reaktionen zusammenhängt. Gestützt auf die Ergebnisse beider Versuchsreihen wird eine Deutung des Temperatureffekts gegeben. Dabei wird die Gesamtaktivierungsenergie aufgespalten in zwei Komponenten von 0,3 kcal/Mol für die Überwindung sterischer Behinderung am Muttermolekül und von 0,65 kcal/Mol für die Aufrechterhaltung der Diffusion durch Freisetzung der Radikale aus Potentialmulden.

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