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# Inactivation of Bacteriophage, DNA, and Ribonuclease by Thermal Hydrogen Atoms

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T1 phage, BU-T1 phage, infectious DNA extracted from phage $\phi$X 174, and chromatographically purified ribonuclease were exposed to thermal hydrogen atoms, and the loss of plaque-forming ability, infectivity, or enzymatic activity was determined after various exposure times. Atomic hydrogen was generated by two different methods: (1) by a high-frequency discharge in hydrogen gas and (2) by irradiating a foil of polyethyleneterephthalate with 2-MeV protons. With increasing exposure time the surviving fraction of all objects tested approaches a constant level. After subtracting this constant "indestructible" fraction in either system, all objects were inactivated according to exponential curves. Furthermore, no BU sensitization was found to occur in BU-T1 phage exposed to atomic hydrogen, whereas gamma irradiation of samples from the same batches revealed a BU effect of a factor of 2.2. These experiments demonstrate hydrogen atoms to be efficient in causing biological damage. Consequently the terminology of "direct" and "indirect" radiation effect may have to be redefined.

## INTRODUCTION

Since Bonhoeffer's fundamental experiments during the twenties, it has been known that hydrogen atoms react with numerous inorganic and organic molecules containing hydrogen and that in many of these reactions abstraction of hydrogen occurs (*1*, *2*). Subsequently the action of H atoms was investigated in quite a number of chemical systems. The monograph by Steacy (*3*) gives a comprehensive survey of the literature published up to 1953. During the past years increasing interest has been focused on the problem concerning the contribution of atomic hydrogen to the radiation inactivation of macromolecules of biological importance. This interest was stimulated by the evidence that in irradiation of hydrocarbons

(*4*), amino acids,[1] proteins (*5*, *6*), and nucleic acid constituents (*7–9*) a gas is formed, the major fraction of which is molecular hydrogen. Furthermore, it could be concluded from electron spin resonance (ESR) experiments that many radicals of irradiated organic molecules are formed by breakage of C—H bonds (*10*). In 1964 Patten and Gordy (*11*) showed that hydrogen atoms were generated by irradiating frozen aqueous solutions of nucleic acids at 4.2°K. The H atoms were stabilized by trapping, thus enabling their detection by ESR. Recently Müller and Dertinger (*12*) described similar experiments using highly purified preparations of phage T1, T2, T4 $Bo^r$, and phage DNA. The radiation-induced hydrogen atoms generated and trapped in the dry material at 77°K showed a relatively high stability which follows from the fact that warming up to 200°K for 10 minutes reduced the amplitude of the H-lines in the ESR spectrum to half its original value only.

In 1963 Braams (*13*) suggested a mechanism for the action of ionizing radiation which explained numerous experimental findings obtained after irradiation of proteins and nucleic acids in the dry state. According to this hypothesis a macromolecule, MH, under irradiation dissociates into a macroradical, $\dot{M}$, and a hydrogen radical, $\dot{H}$:

$$MH \rightarrow \dot{M} + \dot{H} \tag{1}$$

Hydrogen atoms are very potent chemically; furthermore H atoms generated by irradiation often are electronically excited (*14*), as can be seen from the fact that the emission lines of atomic hydrogen are observed when molecules containing hydrogen are irradiated with fast electrons (*15*). In any case the H atoms liberated possess sufficient energy to react with undamaged molecules, MH, by hydrogen abstraction:

$$\dot{H} + MH \rightarrow \dot{M} + H_2 \tag{2}$$

Part of the molecular hydrogen observed in irradiated organic compounds (cf. references *4–11*) may be generated according to reaction 2, as well as by recombination of hydrogen atoms. The macroradicals $\dot{M}$ may undergo further reactions by forming cross-links of the following type:

$$\dot{M} + \dot{M} \rightarrow M—M \tag{3}$$

$$\dot{M} + MH \rightarrow M—M + \dot{H} \tag{4}$$

In reaction 4, besides the dimers M—M, additional hydrogen atoms are produced which participate in further reactions according to equation 2. In this scheme the protective effect of SH compounds (RSH) or substances containing -SS- linkages ($R_1SSR_2$) is referred to an unspecific scavenging of H atoms. By this process the total number of macromolecules damaged in accordance with equation 2 is diminished:

$$\dot{H} + RSH \rightarrow R\dot{S} + H_2. \tag{5}$$

$$\dot{H} + R_1SSR_2 \rightarrow R_1SH + R_2\dot{S} \tag{6}$$

[1] J. J. ten Bosch and R. Braams, unpublished results, cited by Braams (*13*).

To explain the protection factors of about 2 frequently obtained in irradiated dry systems, one has to suppose that reaction 2 not only takes place but also affects the specific properties of biomolecules It is known indeed that in aqueous solutions hydrogen atoms destroy the activity of enzymes (*16*, *17*) and the plaque-forming ability (PFA) of phage T7 (*18*). For dry systems it has been shown by ESR measurements that H atoms react with proteins (*19*) as well as with DNA and its constituents (*20–27*), but up to now no experimental proof has been available that this reaction leads to an inactivation of dry enzymes, DNA, and bacteriophage. In addition, it has not yet been established that such a reaction proceeds with the same kinetics as does inactivation by irradiation. If the inactivation curves obtained with hydrogen atoms and ionizing radiation were different in shape, it could be concluded that H atoms do not substantially contribute to the damage observed in irradiated biomolecules as postulated by equation 2. It was shown in a preceding paper (*28*) that the major fraction of ribonuclease is converted into dimers by irradiation in the dry state *in vacuo*, under oxygen atmosphere, and at 77°K, thereby giving experimental evidence for equations 3 and 4; it is the purpose of the present work to determine the validity of equations 1 and 2 by measuring the effect of atomic hydrogen on various "biological" specimens.

## EXPERIMENTAL METHODS

### *Bacteriophage*

Bacteriophage T1 was grown in M9 medium, precipitated with ammonium sulfate, purified according to the method of Herriott and Barlow (*29*) as modified by Hotz (*30*), concentrated in the CsCl gradient, and finally dialyzed against 0.25 *M* Tris buffer (pH 8.1) to remove CsCl. Suspensions of adequate phage concentration were diluted 1:1000 with triple-distilled water, the pH of which previously had been adjusted to 7.6 by addition of ammonia. Experiments showed that dilution $1:10^5$ into ammonia water or 1:1000 into 0.001 % solutions of gelatin or bovine serum albumin (BSA) did not affect the sensitivity of T1 to H atoms. Substitution of thymine by the base-analogous 5-bromouracil (BU) was achieved according to a method described by Stahl *et al.* (*31*). Quantitative analysis of base substitution as described by Hotz (*32*) showed about 65 % of the thymine to be replaced by bromouracil (BU). BU-T1 phage was purified, concentrated, diluted, and freeze-dried in exactly the same way as normal T1.

### *Sample Preparation*

The small penetration depth of atomic hydrogen requires samples to be exposed in very thin and homogeneous layers. For this purpose 0.01 ml of diluted phage suspension was pipetted onto microscope cover slips 12 mm in diameter, frozen on a brass block cooled to −78° C, and finally lyophilized at $10^{-2}$ torr while the temperature of the samples was slowly increased. By this procedure plaque-forming ability (PFA) is reduced by a factor of about 2. After H atom exposure or irradiation, bacterio-

phage was removed from the cover slips by immersion in 1 ml of tryptone broths and the fraction of surviving phage was determined by plating on *Escherichia coli* K12.

*Infectious DNA*

Bacteriophage $\phi$X 174 was prepared and purified by the method of Sinsheimer (*33*). The single-stranded DNA was isolated by three successive extractions with hot phenol saturated with buffer, as described by Gutherie and Sinsheimer (*34*). The phenol was removed with ether, and the ether was expelled by bubbling nitrogen through the solution. The DNA was then dialyzed against 0.2 *M* NCE buffer (0.179 *M* NaCl, 0.02 *M* Na-citrate, 0.001 *M* EDTA). After dilution of the DNA $1:10^3$ into triple-distilled water (pH 7.6 by $NH_4OH$), 0.01 ml of the diluted solution was freeze-dried on cover slips as described above. With DNA, too, the fraction surviving lyophilization amounted to about 50%.

*Spheroplasts*

Spheroplasts were prepared from a strain of *E. coli* K12 obtained by Dr. G. Hotz from Dr. P. Starlinger, Cologne, in 1962. The method used in our experiments is a modification of the procedure described by Guthrie and Sinsheimer (*34*). The bacteria were grown in 3XD medium (*35*) to a concentration of about $10^9$ cells/ml. Eighty milliliters of cell suspension was centrifuged for 15 minutes at 5000 rpm. The sediment was resuspended in 1.4 ml of 1.5 *M* sucrose and incubated for 15 minutes at room temperature after addition of 0.68 ml of BSA (30%), 0.08 ml of lysozyme (2 mg/ml dissolved in 0.25 *M* Tris buffer, pH 8.1), 0.16 ml of EDTA (4%), and 10 ml of PAM medium (*34*). The reaction was stopped by adding 0.8 ml of $MgSO_4$ (10%). The protoplast suspension obtained by this procedure is used for infection without further treatment and remains usable for several weeks when stored in the refrigerator. By omitting the lengthy procedure of washing the protoplasts by sedimentation into a column of several layers having different densities, as required by the original method, the time necessary for a DNA assay is appreciably reduced, For infection the DNA freeze-dried on cover slips was resuspended in 0.5 ml of Tris buffer (0.05 *M*, pH 8.1), then incubated at 37°C with 0.5 ml of protoplast suspension for 20 minutes, and after addition of 1 ml of PAM medium (of 37°C) for another 100 minutes. After appropriate dilution with Tris buffer, aliquots of 0.1 ml were plated on *E. coli* C/1. A calibration curve for the method used in these experiments is given in Fig. 1. The DNA solution obtained after dialysis against NCE buffer was diluted $1:10^3$ to $1:10^9$ into Tris buffer, and aliquots of 0.5 ml were used for infection. As shown on Fig. 1 the number of plaques obtained is linear with $\phi$X-DNA concentration over at least six decades.

*Ribonuclease*

Pure ribonuclease A was separated from commercially available RNase by chromatography on Amberlite IRC-50 (XE 64). The procedures of purifying, desalting,

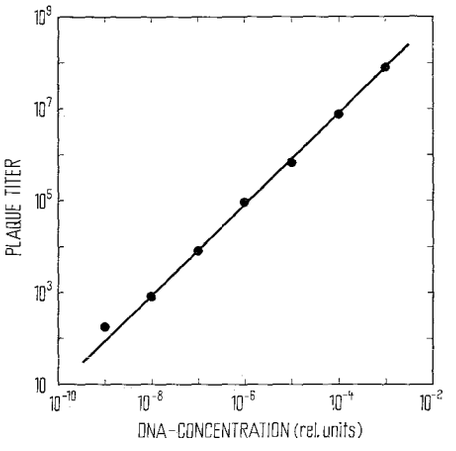


FIG. 1. Calibration curve for the assay of infectious DNA extracted from phage $\phi$X 174. The number of infective centers per milliliter at the end of the infection period (= plaque titer) is given as a function of relative DNA concentration.

and testing the homogeneity of the purified enzyme are described in a previous paper (*36*). A ribonuclease solution (0.01 ml) containing 50 $\mu$g/ml was pipetted onto glass cover slips and lyophilized. After H atom exposure the RNase was dissolved off each cover slip by immersion in 1 ml of acetate buffer (0.1 *M*, pH 5.0), and the reaction between enzyme and substrate was started by adding 1 ml of 1% Na-RNA solution (in 0.1 *M* acetate buffer, pH 5.0). The enzyme assay was carried out as previously described (*37*).

*Irradiation*

Gamma irradiations were performed in a $^{60}$Co gamma source (Gamma-cell 220) at a dose rate of 1.3 Mrads/hour. Several cover slips were placed in a wire coil and sealed with a flame in glass ampules after being evacuated for several hours at $10^{-5}$ torr. Ultraviolet irradiation was carried out with a low-pressure mercury lamp (Hanau, model 6/20) emitting light of 2537 Å mainly. As the relative sensitivities of phage T1 and BU-T1 were to be compared, no absolute dosimetry was necessary.

*Hydrogen Discharge*

To study the action of atomic hydrogen on various specimens of biological importance, two different arrangements were employed. System I consists in an electrodeless discharge in a stream of hydrogen gas. Figure 2 shows the apparatus used: By applying a high voltage of high frequency (30 kV, 30 kc/sec) to two ring electrodes, a discharge was sustained in hydrogen gas passing through a glass tube 5 mm wide at a constant pressure of 16 torr. As the distance between the electrodes and the samples is about 10 centimeters, charged components generated in the dis-

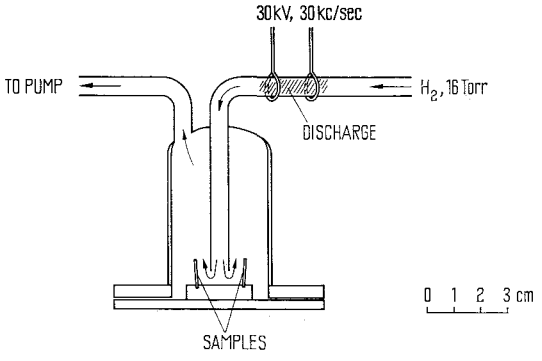


FIG. 2. Setup for exposing specimens to atomic hydrogen generated in a gas discharge (system I).

charge have sufficient time for recombination. The mixture of atomic and molecular hydrogen passes over the surface of the samples to be inactivated. Special care is necessary to make sure that no UV light arising from recombination can reach the the samples. By applying the relatively high gas pressure of 16 torr the discharge does not burn beyond the electrodes, whereas at lower pressure it would extend almost down to the samples. But even at 16 torr sufficient UV light originates from recombination in the perpendicular part of the discharge tube to inactivate samples placed beneath the muzzle of the discharge tube. Therefore the samples were attached vertically in such a way as to minimize the volume from which UV photons originate. In this arrangement the contribution of UV light to the inactivation of the samples exposed to hydrogen atoms is minimized and within the limits of experimental error (cf. Discussion). Absolute dosimetry was not performed. However, the discharge was sufficiently constant to guarantee a linear correlation between dose and exposure time.

*"Condenser" Experiment*

System II permitted the separate investigation of the two postulated reactions according to equations 1 and 2. In a setup having some similarity to a parallel-plate condenser, a foil of polyethyleneterephthalate (trade names: Hostaphan, Melinex, or Mylar) 10 microns thick is facing the samples to be exposed to hydrogen atoms. The arrangement shown in Fig. 3 is attached to the exit port of a van de Graaff machine and is maintained at a pressure of $5 \times 10^{-6}$ torr. Protons (2 MeV) pass through a collimator system and hit the foil at an angle of 9 degrees. Hydrogen atoms are liberated from the surface of the foil by irradiation and react with the specimens on the opposite side of the condenser. Absolute dosimetry was not performed. However, the variation in the intensity of the proton beam impinging on the foil was so small that the number of hydrogen atoms reaching the samples could be assumed to

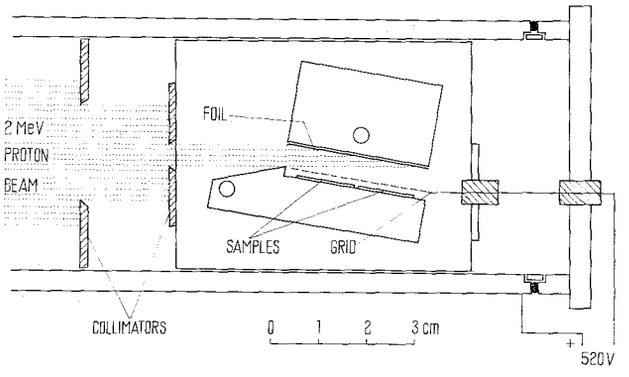


FIG. 3. Setup for exposing specimens to atomic hydrogen generated by bombarding a foil of polyethyleneterephthalate with 2-MeV protons (system II).

be proportional to irradiation time. Secondary electrons liberated by the protons are kept off the samples by a grid held at a negative potential of 520 volts. The probability for a proton scattered from the primary beam to leave the foil is smaller than $10^{-7}$, as can be calculated by multiple scattering theory (*38*). Furthermore, our results prove beyond doubt that the contribution of secondary electrons and backscattered protons to the inactivation observed may be neglected within experimental error (cf. Discussion).

## RESULTS

Figure 4 shows a typical inactivation curve of the kind obtained with all objects investigated in systems I and II. In the example given, the plaque-forming ability of BU-T1 phage is plotted versus time of exposure to hydrogen atoms generated by method II. With increasing exposure time the surviving fraction approaches a constant value indicating that the thickness of the irradiated samples exceeds the diffusion length of hydrogen atoms in some places, which means that part of the material is not accessible to H atoms. The height of this constant value depends on the kind of preparation and amounted to 12 to 35 % in different experiments. For each inactivation curve this value was determined with good statistics by long time exposure of 10 to 15 samples. After subtraction of this constant fraction, the experimental values fall on an exponential inactivation curve (cf. Fig. 4) the slope of which, represented by the $D_{37}$, is a measure for the relative sensitivity of the specimens inactivated.

Figure 5 shows dose-effect curves for phage T1 and BU-T1 inactivated by hydrogen atoms generated in systems I and II. The experimental values originating from at least two independent runs are plotted by taking the number of phage surviving lyophilization minus constant value to be 100 %. It should be noted that the results for T1 and BU-T1 fall on the same straight line, which means that substitution of

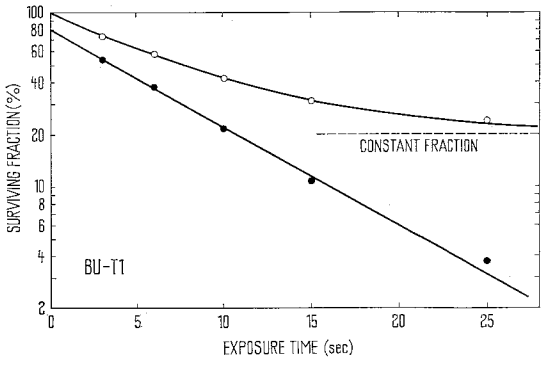


FIG. 4. Typical inactivation curve obtained after exposure of phage BU-T1 to atomic hydrogen (system II).

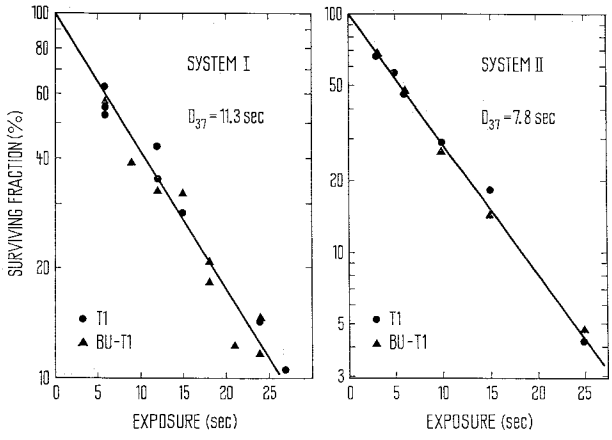


FIG. 5. Inactivation of dry phage T1 (●) and BU-T1 (▲) by exposure to atomic hydrogen (systems I and II).

thymine by bromouracil does not alter the sensitivity of T1 phage to atomic hydrogen. The $D_{37}$ values taken from the graph amount to 11.3 and 7.8 seconds. The fact that these values obtained in either system happen to be of the same order of magnitude is, of course, accidental.

In Fig. 6 the dose-effect curves for the inactivation of infectious $\phi$X 174-DNA by H atoms are plotted. For single-stranded DNA, too, in systems I and II after subtraction of the constant fraction, exponential inactivation curves are obtained the slope of which is somewhat smaller compared to the inactivation curve of phage T1.

Figure 7 shows the reduction in enzymatic activity of ribonuclease when treated with atomic hydrogen. The $D_{37}$ exposure times are 12.9 minutes for system I and 8.5 minutes for system II.

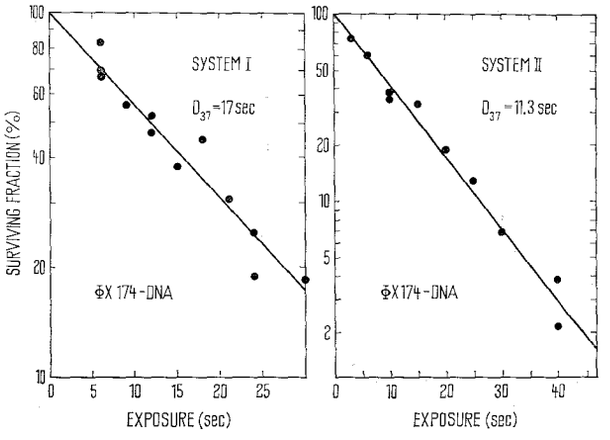


FIG. 6. Inactivation of dry infectious $\phi$X 174-DNA by exposure to atomic hydrogen (systems I and II).

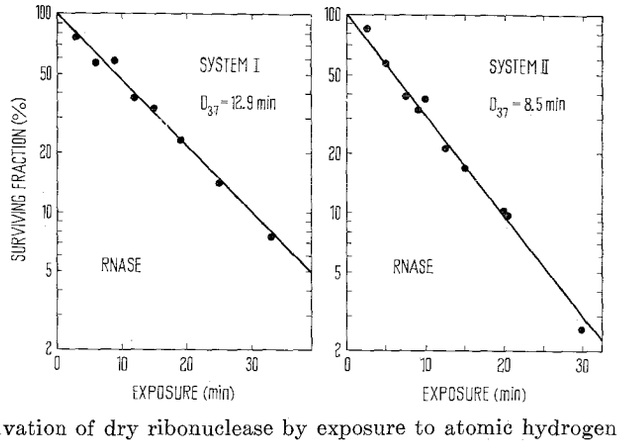


FIG. 7. Inactivation of dry ribonuclease by exposure to atomic hydrogen (systems I and II).

For comparison, irradiations were carried out under anaerobic conditions with $^{60}$Co gamma radiation. The 37% doses determined were: T1 phage 320 krads; BU-T1 phage 145 krads; $\phi$X 174-DNA 320 krads; and RNase 42 Mrads (*39*). When lyophilized samples are irradiated with UV light of 2537 Å, the difference in the sensitivity of T1 and BU-T1 amounts to a factor of about 3 in the first decade of inactivation.

## DISCUSSION

Figure 8 summarizes the dose-effect curves for the inactivation of T1 and BU-T1 by various methods, revealing one interesting result of the present work: the absence

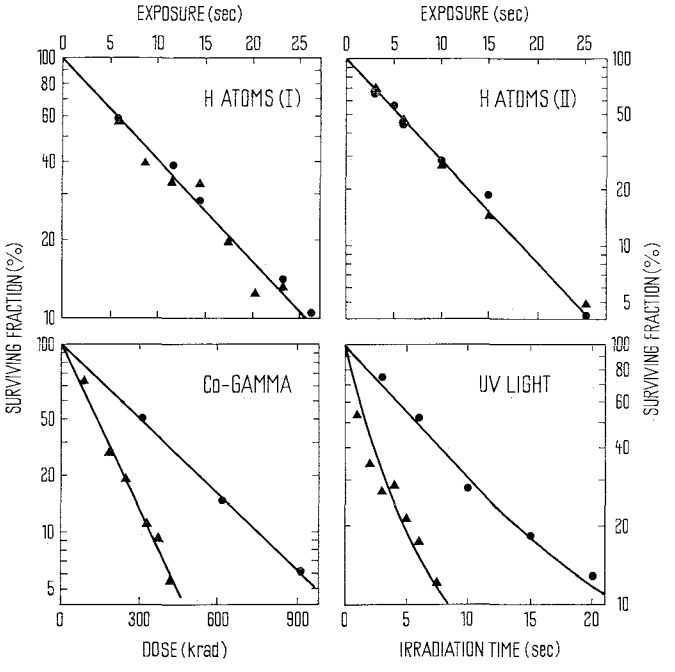


FIG. 8. Inactivation of phage T1 (●) and BU-T1 (▲) by exposure to atomic hydrogen in systems I and II and by irradiation with $^{60}$Co gamma radiation and with UV light of 2537 Å.

of BU sensitization to the action of hydrogen atoms. With gamma radiation the sensitivity of BU-T1 phage is higher by a factor of 2.2 as compared to that of normal T1 phages. With UV light in the first decade the two inactivation curves for T1 and BU-T1 differ in slope by a factor of about 3. This sensitization factor observed with lyophilized phages is comparable with that found by Hotz and Zimmer (*40*) with phage suspended in 0.8% NaCl solution. However, phage T1 and BU-T1 are inactivated by H atoms with identical velocity as found with systems I and II. From these results conclusions may be drawn concerning the reliability of our experimental arrangements: If UV light did play a major role in damaging samples exposed to H atoms from the hydrogen discharge, a BU effect should be observed in system I; and if secondary electrons or scattered protons were responsible for an appreciable portion of the inactivation found in system II, BU sensitization would be expected in the "condenser" experiment. Thus, our findings provide clear evidence that the inactivation observed in either system is due to the action of atomic hydrogen, ruling out the possibility that UV light or charged partices contribute to the observed effects to any appreciable extent.

One can think of several hypotheses to explain the absence of a BU sensitization in the action of hydrogen atoms on T1 phages:

1. The reactivity of H atoms may be so high that they are unable to penetrate through the protein coat. Corresponding to this assumption, inactivation of phage is initiated by producing damage in the protein coat (for example, a radical site) which migrates to the DNA or by affecting the adsorption mechanism in the phage tail. In either case the sensitivities of T1 and BU-T1 are expected to be identical, as the phage differ with regard to their DNA composition but not their protein coat. This problem may be studied by inactivating dry BU-DNA from phage $\phi$X 174 by atomic hydrogen and comparing its sensitivity with that of normal $\phi$X-DNA. An experiment of this kind is in preparation.[2] Unfortunately it is not yet possible to compare the sensitivities of $\phi$X 174 phage with infections $\phi$X-DNA or of T1 phage with infectious T1-DNA, as $\phi$X phage is inactivated during lyophilization in the absence of organic material and the assay for infectious T1-DNA is not yet possible with reasonable efficiency.

2. A second hypothesis to explain the nonexistence of a BU effect for H atoms is based on the supposition that atomic hydrogen penetrates through the protein coat and does inactivate the phage by damaging its DNA. As shown by Heller and Cole (*20*) in thymine treated with atomic hydrogen, exclusively the $C_{(6)}$ addition radical is formed which has a characteristic ESR pattern consisting of eight well-resolved lines. Exposure of DNA to H atoms mainly gives rise to a signal centered near the $g$ factor for the free electron which has great similarity with the ESR spectra obtained with adenine, guanine, and cytosine. This result may indicate that in the dry state the rate constant of H atoms for the reaction with thymine is smaller than those for the reaction with the other DNA constituents, although this is not found for the rate constants measured in water (*41*). Under these assumptions the replacement of thymine by bromouracil will not affect the overall reaction rate of DNA, but we do not think this interpretation to be very probable.

3. A third interpretation is based on the assumption that in the action of gamma radiation and UV light bromine radicals are liberated from BU-DNA by relatively small amounts of energy which in thymine-DNA are only sufficient to cause excitation. By this process more primary base damage is caused per unit dose in BU-DNA than in normal DNA. This interpretation is in line with the findings by Müller *et al.* (*42*) showing in bromouracil the production of ESR centers per unit dose to be higher by almost an order to magnitude than in thymine. Additional evidence from photochemical and radiochemical studies indicates that uracil is the main product formed when bromouracil-substituted DNA is irradiated with UV light or x-rays, respectively (*43*, *43*). Furthermore, the bromine radicals split off may react with undamaged molecules, thus increasing the overall inactivation observed.

It is not yet possible to descide which of the interpretations suggested above represents the reason for the absence of a BU sensitization in the action of H atoms. This is not too surprising, as even for gamma radiation and UV light, which up to now have been applied in a great number of experiments using phage with normal

[2] K. Kürzinger, H. Jung, and G. Holtz, in preparation.

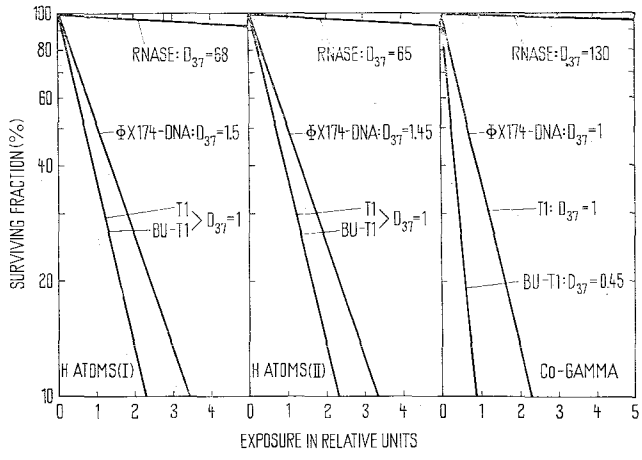


FIG. 9. Inactivation curves obtained for T1, BU-T1, infectious $\phi$X 174-DNA, and ribonuclease by exposure to atomic hydrogen (systems I and II) and by $^{60}$Co gamma irradiation, $D_{37}$ exposure for T1 being normalized to unity.

and BU-substituted DNA, no generally accepted explanation for the molecular mechanism of BU sensitization has been worked out. Therefore our results regarding the differences of BU sensitization in the action of ionizing radiation and atomic hydrogen provide additional information to check the reliability of pertinent hypotheses, for any general hypothesis should also be able to describe why only the actions of ionizing radiations and UV light may be modified by incorporation of bromouracil into DNA, whereas with hydrogen atoms such a BU sensitization is not observed.

Our findings are summarized in Fig. 9, where the relative sensitivities of all objects investigated are compared, omitting the single measured points for clarity. The curves are normalized to a $D_{37} = 1$ for phage T1. In either system BU-T1 phage have the same sensitivity ($D_{37} = 1$) as phage T1, whereas for infectious $\phi$X-DNA the relative $D_{37}$ is 1.5 in system I and 1.45 in system II. The corresponding values for ribonuclease are $D_{37} = 68$ with method I and $D_{37} = 65$ with method II. The good agreement of the results obtained with either experimental arrangement indicates identical mechanism of inactivation to act. This conclusion is supported by comparing the action of H atoms with the results of our experiments using gamma radiation. Here the relative $D_{37}$ for BU-T1 amounts to 0.45 corresponding to a sensitization factor of 2.2. The relative $D_{37}$ for the inactivation of infectious $\phi$X-DNA by gamma rays ($D_{37} = 1$) is smaller than the value obtained with H atoms, and for ribonuclease we found $D_{37} = 130$, indicating RNase having a higher sensitivity to H atoms than to gamma rays as compared to T1 phage.

In a preceding publication (*45*) we gave some indication that diffusible radicals

may contribute to the inactivation of biological specimens irradiated with ionizing radiation. In this paper we showed that the cross section for the inactivation of ribonuclease by 2-MeV protons consists of three components, one of which is independent of temperature while the two others have apparent activation energies of 1 kcal/mole and 6.5 kcal/mole. Similar values were derived from the temperature dependence of the radiosensitivity of amino acids, enzymes, single- and double-stranded phage, and spores of *Bacillus megaterium* (*39*). On the basis of these relatively small activation energies, we concluded that the inactivation process involved might, at least partly, consist of radical reactions (*39*, *45*). This hypothesis is supported by the findings of the present work, especially by the fact that all objects subjected to hydrogen atom exposure are inactivated according to exponential curves. As this inactivation kinetics is the same as was found in experiments with gamma radiation, our results are in line with the scheme given earlier which ascribes part of the biological effects observed in dry systems after irradiation with ionizing radiation to the action of hydrogen atoms. Additional information regarding the meaning of our results for molecular radiobiology may be found in a review article by Zimmer *et al.* (*46*).

Furthermore, our experiments demonstrate that the definition of "direct" and "indirect" radiation action as used up to now may have to be redefined. For about three decades the actions of radiations on dry systems have often been subsumed under the name "direct effect", on the assumption that in the dry state only such molecules are affected as have interacted directly with the primary or secondary radiation. Experimental proof is now available that H atoms are liberated in various biomolecules by irradiation and are also capable of inactivating such entities. Thus, in the nomenclature used one should take into consideration that two mechanisms of inactivation occur in irradiated dry systems: Only part of the material inactivated is altered by direct absorption of radiation energy, and it may be useful to restrict the term "direct effect" to describing this kind of damage. Another portion of the specimens is affected by secondary radicals liberated by irradiation, and these radical processes should be characterized in an adequate manner.

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