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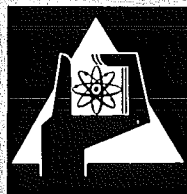
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Formation of hydroxyl radicals in thymine solution by excited hydrogen or argon from a gas discharge

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The effects of hydrogen gas excited in a radiofrequency discharge on oxygen-free solutions of thymine have been followed chemically. Two main reaction products have been identified: 5,6-dihydrothymine and 5,6-thymine glycol. The latter product was found to be the only significant species if discharge-excited argon was pumped through the solutions instead of hydrogen. Although dihydrothymine clearly is the reaction product of atomic hydrogen generated in the hydrogen discharge, the glycol requires the formation of OH radicals in the solution. A mechanism is proposed by which this species is produced through (super)excitation and decay of water molecules by energy transfer from the excited gas.

1. Introduction

Since it has become evident that hydrogen atoms (H radicals) are responsible for a considerable amount of radiation damage to biological objects (see Dertinger and Jung 1970), numerous experiments have been performed to study selectively the action of atomic hydrogen on biomolecules. The experimental information about H radical attack comes from E.S.R. studies in the dry state (Herak and Gordy 1965, Holroyd and Glass 1969), from biological inactivation experiments in the dry state (Jung and Kürzinger 1968), and in aqueous solutions (Mee, Navon and Stein 1965, Dewey and Stein 1968, Jung, Hagen, Ullrich and Petersen 1969). The hydrogen discharge was applied in most of these experiments as an efficient source of H radicals. However, in spite of this widespread application of the discharge, there is a lack of experiments involving a more rigorous analysis of the products that are actually formed when pumping discharge-excited hydrogen gas through aqueous solutions of biomolecules. The present study therefore aimed at elucidating the effects and mechanisms that cause inactivation. Thymine was chosen as the test molecule, since it represents a compound of great biological interest and has been well examined as a preferential site of radical attack in deoxyribonucleic acid.

2. Materials and methods

2.1. The gas discharge apparatus

The gas to be excited was allowed to pass through a pressure-reducing valve and a needle valve into the evacuated quartz discharge tube, from where it was pumped through the reaction vessel containing the cooled solution (5°C). A trap cooled in liquid air was inserted between the needle valve and the discharge tube to freeze out traces of water vapour and other impurities that might be present.

The discharge was maintained by a 40 MHz-generator (30 Watts) coupled to the discharge tube by external electrodes. Precautions were taken to prevent u.v.-light generated in the discharge from reaching the solution: a high operating pressure of 50 Torr, as well as the insertion of a 10 cm-long bent tube between discharge and reaction vessel. The gas bubbled into the solution through perforations (0.1 mm in diameter) at the end of the tube. An attempt to detect free radicals with E.S.R. after exposure of dry thymine to discharge-excited argon under these experimental conditions gave negative results, whereas direct u.v.-irradiation was found to be very effective in producing thymine radicals. Thus, the contribution of u.v.-light to the results of this investigation can be neglected. The gas stream was adjusted to approximately 600 l./min. Hydrogen and argon used in these experiments were of high purity. To remove dissolved oxygen, the solutions were bubbled with hydrogen and argon, respectively, for a period of 5 min before ignition of the discharge.

2.2. Separation and identification of the reaction products

Chromatographically pure 2-¹⁴C-thymine (23.9 μ Ci/mg) was used, and the reaction products were separated and determined by thin-layer radiochromatography, using a Berthold thin-layer scanner. For this purpose, 5 ml. samples were taken from the exposed 10⁻³ M thymine solutions (pH \sim 6.5) and concentrated 10 times at 30°C under reduced pressure; 10 μ l. (\sim 0.3 μ Ci) were then chromatographed, using precoated plates of silica gel F₂₅₄, cellulose F (Merck) or chromatography paper Whatman No. 1. The following eluents were applied (composition in v : v):

A: *n*-butanol/water (86 : 14);

B: *n*-propanol/water (10 : 3);

C: ethylacetate/water/formic acid (60 : 35 : 5);

D: *n*-butanol/methanol/water/NH₄OH 28 per cent (60 : 20 : 20 : 1)

Only solvent A was used for paper chromatography (PC), whereas for thin-layer separation assays on CTL (cellulose thin layer) and GTL (silica gel thin layer), the other eluents were also applied. The best separation could be obtained with CTL and solvent C. The relative quantity of the reaction products was determined by graphic integration of the chromatographic ¹⁴C-peaks. The reproducibility of the whole method was better than \pm 15 per cent.

The reaction products were tested with respect to saturation of the 5,6-double bond of thymine (T), either directly on the plates or after enrichment; α -diol groupings (T(OH)₂) were identified by the characteristic reaction with meta-periodate and subsequent detection with benzidine (Cifonelli and Smith 1954). In addition, use was made of the blue colouring after the reaction of T(OH)₂ with *p*-dimethylaminobenzaldehyde (*p*-DAB) subsequent to alkaline treatment (Teoule and Cadet 1970); with dihydrothymine (TH₂) *p*-DAB gives a yellow colour under the same conditions (Fink, Cline, McGaughey and Fink 1956).

2.3. Detection of oxidizing and reducing species

10⁻² M solutions of KI were used for the identification of OH radicals. The oxidation to iodine was followed by measuring photometrically at 565 nm the blue starch-gel colouring against calibrated iodine solutions. By an analogous procedure, hydrogen peroxide generated in doubly-distilled water

could be determined quantitatively simply by adding KI and starch gel after exposure and measuring the colouring against calibrated H_2O_2 solutions.

Reducing species, such as electrons and H radicals, were determined by the reduction of $\text{K}_3[\text{Fe}(\text{CN})_6]$ in 10^{-2} M solutions. The $[\text{Fe}(\text{CN})_6]^{4-}$ ions generated in this way give with FeCl_3 the Berlin-blue colouring, which was measured against calibrated solutions by its absorption at 740 nm.

3. Results

Figure 1 shows the kinetics of thymine destruction as measured by the loss of optical absorption at 264.5 nm.

It is obvious that with discharge-excited argon an effect is also obtained which is comparable in magnitude with that of the hydrogen discharge. Furthermore, it can be seen that the inactivation by hydrogen does not follow a linear dependence throughout the exposure time applied in this experiment. This may indicate that a strong reverse process is acting in the case of hydrogen. Taken as a whole, figure 1 shows that the 5,6-double bond of thymine, responsible for the absorption at 264.5 nm, is affected in both cases.

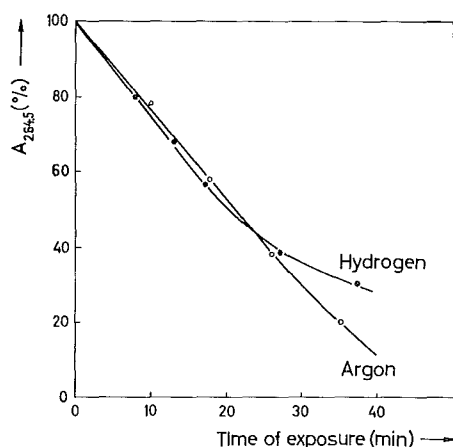


Figure 1. Decrease of absorbance at 264.5 nm of a 10^{-8} M thymine solution exposed to hydrogen or argon from the gas discharge.

Figure 2 shows the CTL-radiochromatograms of thymine solutions after exposure to hydrogen and argon. In figure 3 the product formation is plotted as a function of exposure time. As can be seen, II and IV are the main decomposition products with discharge-excited hydrogen, whereas in the case of argon most of the thymine is converted into product II. Peaks I and III, usually being very small, were not subsequently subjected to further analysis. Compound II and IV do not absorb in the u.v.-region and have to be considered as being 5,6-saturation products of thymine. In fact, product IV has the same running properties in a variety of eluents as the commercially-available compound 5,6-dihydrothymine (table 1) and shows yellow colouring upon reaction with *p*-DAB after alkaline treatment. The appearance of 5,6-dihydrothymine (TH₂) among the reaction products of thymine is, of course, not surprising, since it is formed by the saturation of the 5,6-double bond with two H radicals produced in the hydrogen discharge.

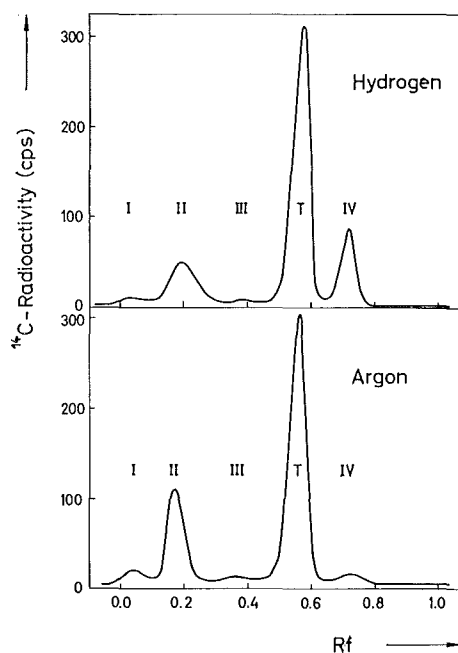


Figure 2. CTL-radiochromatograms with eluent C of 10 μ l. (0.3 μ Ci) 2- 14 C-thymine solution after exposure to hydrogen or argon from the gas discharge. ($A_{264.5} \sim 80$ per cent); run distance of eluent: 14 cm. For the identification of the peaks see text (T=thymine).

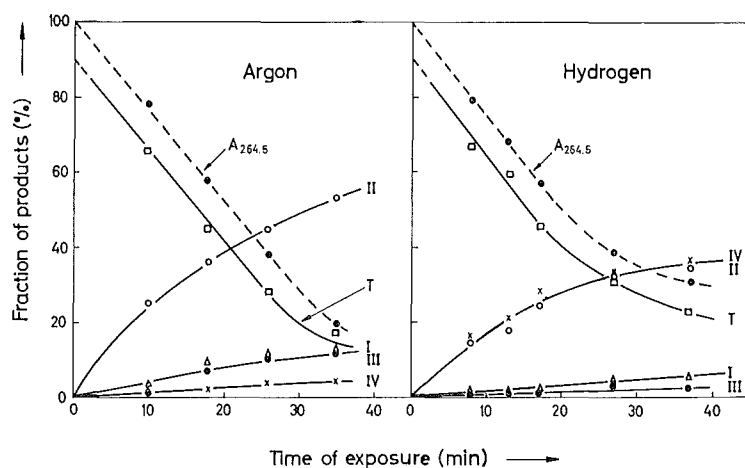


Figure 3. Thymine destruction and product formation in 10^{-8} M thymine solutions as function of exposure to excited argon and hydrogen from the discharge. Sum of products I, II, III, IV (see figure 2) and remaining thymine (T) gives 100 per cent. $A_{264.5}$ = per centage of absorbance of the exposed thymine solution. The overall recovery of thymine after separation reaches about 90 per cent.

Chromatography	Eluent	IV	TH ₂
PC	C	0.61	0.61
CTL	B	0.65	0.66
CTL	C	0.72	0.72
GTL	D	0.79	0.80

Table 1. R_f-values of product IV and 5,6-dihydrothymine (TH₂) in different chromatographic systems. For abbreviations see Methods.

Compound II gives a positive reaction with metaperiodate as well as with *p*-DAB, indicating the presence of an α -diol configuration. Furthermore, the running properties of this substance in different eluents are in close agreement with that of *cis*-thymine glycol, synthesized according to the method of Baudisch and Davidson (1925). This is shown in table 2. Thus the compound *cis*-5,6-dihydroxythymine (thymine glycol, T(OH)₂) is compatible with the results of the analysis of product II. It should be mentioned that peak II is usually found to be asymmetrical (figure 2). It is assumed that partial conversion of the *cis*-form into the *trans*-form, resulting in a change of the running properties, is responsible for this effect (Teoule and Cadet 1970). Clearly the formation of compound II requires the attack of OH radicals. We therefore attempted directly to identify this species through its oxidizing properties. The oxidation of KI with subsequent identification of iodine (see Methods) was chosen as test reaction. To be sure that no H radicals interfere with this reaction, this experiment was performed with the argon discharge. Figure 4 (*a*) shows that there is a linear increase in iodine concentration with exposure time. It should be mentioned that in the presence of I⁻ ions the iodine occurs as I₃⁻. However, in spite of this 'stabilization', part of the iodine unavoidably sublimates out of the solution at 50 Torr. Thus the yield of OH[•] measured by this method is probably smaller than the actual value.

As another independent proof of OH radical formation, the production of hydrogen peroxide resulting from combination of two OH radicals was measured. For this purpose, doubly-distilled water was bubbled with discharge-excited

Chromatography	Eluent	II	T(OH) ₂	T
PC	A	0.20	0.21	0.50
CTL	A	0.20	0.20	0.51
CTL	B	0.40	0.40	0.63
CTL	C	0.19	0.20	0.57
CTL	D	0.39	0.39	0.62
GTL	B	0.65	0.65	0.75

Table 2. R_f-values of product II, *cis*-5,6-dihydroxythymine (T(OH)₂) and thymine in different chromatographic systems. For abbreviations see Methods.

hydrogen or argon and samples taken at various time-intervals. It is evident from figure 4 (b) that KI added to the samples is oxidized to iodine (see Methods) due to the formation of H_2O_2 .

The presence of OH radicals may explain the observed non-linearity in the kinetic curve for the hydrogen discharge (figure 1). Since dihydrothymine has been shown to be a major product in this case, abstraction of H atoms from this compound by OH radicals may occur (Myers and Theard 1970), leading to a partial reversion to thymine at higher exposure times.

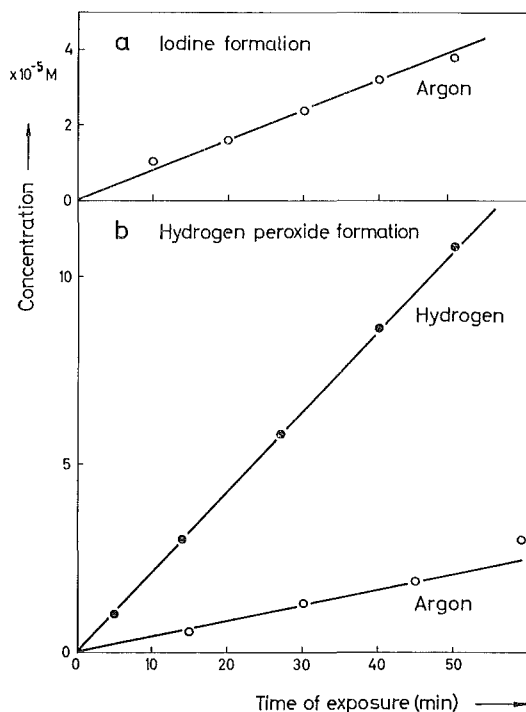


Figure 4. (a) Oxidation of $10^{-2} M$ KI solutions by OH radicals generated during the exposure to excited argon from the gas discharge. The free iodine was measured by the blue starch-gel colouring (see Methods). (b) Hydrogen peroxide formation in doubly-distilled water exposed to hydrogen and argon from the gas discharge as determined by oxidation of KI (see Methods).

Finally, an experiment was carried out to examine whether reducing species are generated in solutions bubbled with discharge-excited gases. This experiment was performed with argon, since in the case of hydrogen the reducing properties of the H radicals generated in the discharge are dominant, so that no statement can be made concerning the generation of reducing species (electrons or H radicals) in the solution. The reduction of $K_3 [Fe(CN)_6]$ solutions with subsequent Berlin-blue test (see Methods) was taken as indication for reducing properties of the solution during exposure. The results presented in figure 5 in fact confirm the presence of reducing species in solutions bubbled with excited argon.

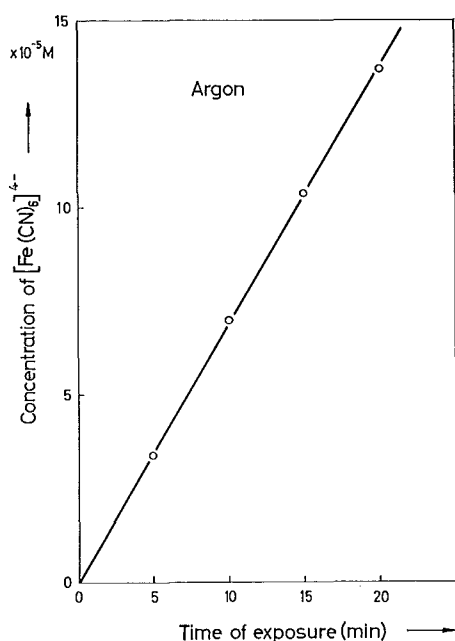
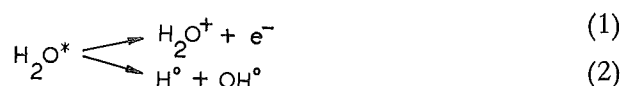


Figure 5. Reduction of 10^{-2} M $K_3(Fe(CN)_6)$ solutions during exposure to γ -excited argon, measured by the Berlin-blue reaction (see Methods).

4. Discussion

The main result of this investigation is the fact that OH radicals are generated, if discharge-excited gases like hydrogen and argon are pumped through aqueous solutions. It has been shown that, in the case of argon, practically the total damage to thymine is due to OH° attack, whereas with the hydrogen discharge additional damage arises from the H radicals generated directly in the discharge. The first question to be discussed refers to the mechanism by which these OH radicals are generated. Electrical discharges through gases produce large amounts of excited atoms and molecules as well as molecular fragments such as H° in the case of hydrogen. Among these species, those excited to long-lived states may reach the reaction vessel and transfer their excitation energy primarily to the water molecules. Since argon and hydrogen have ionization potentials of 15.68 and 15.6 eV, the long-lived states lie below these values, some of them, however, even above the ionization potential of water (12.56 eV). The most probable consequence of transferring excitation energy to water is excitation and even superexcitation of the water molecules. In a simple scheme, the excited water (H_2O^*) is expected to undergo pre-ionization or dissociation:



In the case of pre-ionization (1), H_2O^+ may decay according to:



This means that OH radicals finally occur in both cases. The ratio of the probabilities for dissociation and pre-ionization, which are competitive processes, depends on the amount of energy transferred to the H₂O molecule as may be seen from the excitation spectrum of water published by Platzman (1967). As no precise statements can be made in the present investigation concerning the energy states of the excited gases involved in the energy transfer, the ratio of OH° formation by dissociation and pre-ionization cannot be determined. Since in the case of discharge-excited argon, only negligible amounts of dihydrothymine are formed (figure 2, peak IV), it may tentatively be concluded that pre-ionization is the predominant OH-producing process in this case. However, it must not be overlooked that low-energy electrons are liberated in the process of pre-ionization, part of which might react with the dissolved thymine. The fact that a corresponding thymine reaction product could not be identified by the procedures applied does not exclude an electron reaction. There is evidence that such a product (e.g. a 5- or 6-hydroxydihydrothymine T(HOH)) may be eliminated by some kind of 'reconstruction reaction' (Garrison 1968). If it is assumed that the electron attacks the thymine 5,6-double bond, as do the radicals H° and OH°, then the following reaction proceeds:



Since by an analogous OH° attack the radical TOH° is also formed, TH° and TOH° may react giving:



Upon water elimination, T(HOH) reverts to T (Garrison 1968) so that apparently the e⁻ attack does not necessarily yield a stable reaction product of thymine. That reducing species are in fact present, even in experiments with the argon discharge, is convincingly demonstrated by figure 5. However, it is not clear whether e⁻ or H radicals are responsible for the observed reduction.

It should be mentioned in this context that Czapski, Jortner and Stein (1959) found an oxidizing action of discharge-produced atomic hydrogen, which they ascribed to the species H₂⁺ formed by the reaction of H° with H⁺. However, the results obtained in the present investigation rule out this mechanism as being responsible for the observed oxidizing effects; clearly the H₂O₂ production as well as the fact that also with the argon discharge oxidation is obtained, is not compatible with the H₂⁺ radical.

Summing up the facts, the present study has shown that energy transfer from discharge-excited gases to water with subsequent dissociation or pre-ionization may result in the production of OH°, H° and e⁻. Therefore the hydrogen discharge cannot be regarded as being a suitable source of atomic hydrogen if applied to aqueous systems unless special precautions are taken. The mechanism derived from these results needs, of course, further confirmation by studies comprising other test systems and other methods as well. Investigations of this kind are in progress in this laboratory.

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Ce travail traite de l'influence de l'hydrogène gazeux excité par décharge électrique à haute fréquence sur une solution aqueuse anaérobie de thymine 10^{-3} M. Deux composés, la 5,6-dihydrothymine et la 5,6-dihydroxythymine, représentant la quasi totalité des produits de destruction de la thymine, ont pu être chromatographiquement séparés et identifiés. Le glycol est le principal produit formé lorsque l'argon remplace l'hydrogène. Alors que la formation de dihydrothymine à partir de thymine s'explique facilement par la fixation de l'hydrogène atomique produit par la décharge en milieu H_2 , la formation du glycol nécessite la disponibilité de radicaux hydroxyles dans le milieu réactionnel, ce qui a pu être établi chimiquement d'autre part. Les radicaux OH seraient engendrés par transfert d'énergie du gaz, excité dans la décharge, aux molécules d'eau de la solution, qui se décomposent.

Die Einwirkung von Wasserstoffgas, das einer elektrischen Entladung unterworfen wurde, auf Sauerstoff-freie Thymin-Lösungen wurde chemisch untersucht. Dabei wurden zwei Produkte, die praktisch den gesamten Schaden darstellen, identifiziert: 5,6-Dihydrothymin und 5,6-Thyminglycol. Die entsprechenden Versuche mit einer Argon-Entladung ergaben, daß in diesem Fall der Glycol das ausschließliche Reaktionsprodukt ist. Während die Bildung des Dihydrothymins ohne weiteres als Produkt von H-Radikal-Angriffen verstanden werden kann, erfordert der Glycol die Anwesenheit von OH-Radikalen, die auch nachgewiesen wurden. Es wird ein Mechanismus diskutiert, demzufolge die OH-Radikale durch Energieübertragung von den in der Entladung angeregten Gasen auf das Wasser und anschließenden Zerfall der angeregten Wassermolekeln entstehen.

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