Free Radicals Formed in Uracil and Dihydrouracil by Hydrogen Atoms

RAGNAR BERGENE and THORMOD HENRIKSEN

Department of Biophysics, University of Oslo, Blindern, Norway and Norsk Hydro's Institute for Cancer Research, Montebello, Oslo 3, Norway

Uracil and its dihydro-derivative have been exposed to the products in a hydrogen gas discharge and the induced radicals have been studied with ESR technique. The same radical(s) was formed in both substances. The radical was induced by a simple hydrogen atom addition to the 5 – 6 double bond in uracil and a similar abstraction of a hydrogen atom in dihydrouracil. This reaction may result in two types of radicals depending upon the exact position for the attack of the hydrogen atom. INDO calculations seem to support the model that both radicals are formed. The radical yield depends upon the gas pressure in the discharge tube as well as upon the sample temperature during exposure. Dose-effect curves indicate that the radicals were formed only in a surface layer of the crystalline particles. The observed activation energy for radical formation, 2.0 kcal/mol, probably reflects the diffusion of H atoms into the samples.

In recent years reactive products formed in a hydrogen gas discharge system have been used extensively to induce free radicals in organic substances.[1-11] This experimental technique seemed promising for an extensive study of the effect of thermal hydrogen atoms which are readily formed in the system. It is well known that hydrogen atoms are one of the primary products formed when ionizing radiation is used. However, due to all the other radiation induced products it appears difficult to pinpoint the specific effect of the hydrogen atoms. Consequently, it has been suggested that experiments with hydrogen atoms from a gas discharge may prove useful also in the study of radiation damage to organic compounds.

It is well established that free radicals are formed in biological compounds when exposed to hydrogen gas discharge products. Considerable discussion is, however, going on as to whether these radicals are formed only by the H atoms or if some accompanying discharge product, such as UV-light or excited hydrogen molecules, contribute to a certain extent.[9,12]

In the present work, which is a part of our main study of radiation damage to nucleic acids, uracil and some of its derivatives have been exposed to the

hydrogen gas discharge products and the formation of radicals has been studied by electron spin resonance (ESR) technique. The main purpose is to shed some light on the radical forming processes as well on as the physical behaviour of the induced radicals. Special efforts have been made to obtain quantitative data.

EXPERIMENTAL

Uracil and dihydrouracil were obtained from Sigma Chem. Corp. Freezedried powder as well as polycrystalline particles, selected by a set of standard sieves, were used.

Samples of 20 to 50 mg were exposed to the hydrogen gas discharge products as described in detail in a previous publication.[9] Special care was taken to reduce the UV-light by using a T-shaped discharge tube (*i.e.* the UV-light, including the Lyman α-line of 1216 Å, is mainly trapped in an appendix). With a special exposure vessel the temperature of the sample during exposure could be chosen and set at any desired value between 77 K and 330 K. (This equipment is described in Ref. 11.)

In quantitative studies the number of induced radicals is very often observed *versus* the exposure dose (dose-effect curves). One parameter of particular interest in such experiments is the gas pressure during exposure. It is known that the production of hydrogen atoms in an electrical discharge depends upon the gas pressure and some experimental data have been published.[13-16] However, it seems difficult to obtain reliable experimental data on the dose of hydrogen atoms which reach the sample since the production and subsequent recombination also depend on other factors, such as the geometry and the cleanness of the glass walls. In the present experiments the dose-rate is assumed to be constant when no changes are made in the experimental procedure. In particular, the dose-rate or the amount of reactive products which reached the sample has been measured as a function of the gas pressure in an experiment where the yield of radicals was used as monitor. In such an experiment it is essential to use exposure times (*i.e.* exposure doses) short enough to ensure that saturation effects are not present (see below). The results, using uracil as test substance, are given in Fig. 1. The curve has an unexpected form with a sharp maximum at a gas pressure of approximately 2×10^{-2} torr.
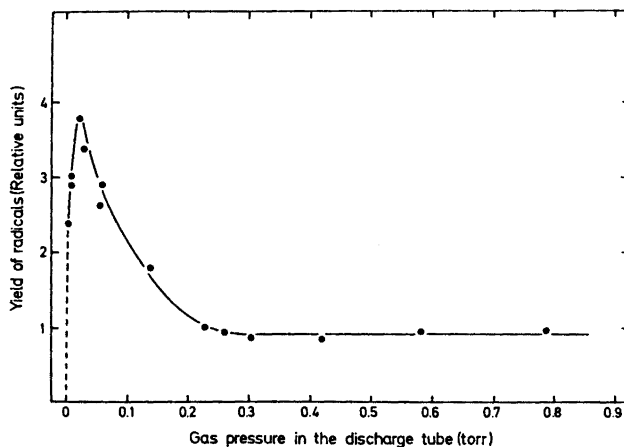

Fig. 1. The yield of radicals induced in uracil as a function of the gas pressure.

Copeland has previously obtained results similar to those in Fig. 1.[17] At present we cannot offer any adequate explanation for the shape of this curve, but due to these results all the subsequent experiments were carried out at a gas pressure of 0.3 to 0.4 torr where small variations in the gas pressure have a negligible effect.

The gas flow through the discharge tube was approximately 3×10^{-5} mol/sec and the exposure time was usually below 1 min. After exposure the sample was transferred to an ESR sample tube, evacuated and sealed off.

The induced radicals were studied with an X-band ESR spectrometer using a transmission cavity and 110 kHz modulation frequency. The details of the spectrometer and the experimental procedure have been described previously.[9-11]

RESULTS

The types of radicals. Uracil and its dihydro-derivative exhibit the same resonance spectrum (Fig. 2) when exposed to the hydrogen gas discharge products indicating that the same radical(s) is formed. A similar spectrum is also obtained when dihydrouracil is exposed to ionizing radiation at room temperature, whereas X-irradiated uracil yields a much more complicated spectrum.
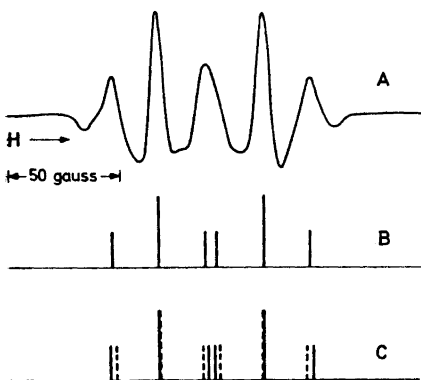
URACIL

Fig. 2. The ESR spectrum of uracil exposed to hydrogen atoms from the gas discharge. Top curve represents the second derivative spectrum observed at liquid nitrogen temperature. The bottom part of the figure shows two stick spectra compatible with the two radical models suggested (see text). Dihydrouracil exhibits an identical resonance spectrum.

The spectrum in Fig. 2 consists of 5 lines with somewhat different intensity and width. The radical responsible for this resonance is probably formed by a hydrogen atom addition to the $5 - 6$ double bond in uracil and a similar abstraction of a hydrogen atom in dihydrouracil. Support for this interpretation emerges from experiments where the hydrogen gas is exchanged with deuterium. Thus, in the case of dihydrouracil the bombardment with deuterium atoms caused no spectral changes as expected, whereas for uracil the spectrum changed according to the addition of a deuterium atom to the double bond. Two different radical candidates (I and II) can be proposed which independently or together will give rise to the resonance in Fig. 2.
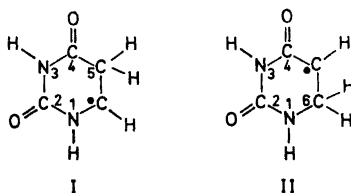
Both the proposed radicals will give rise to a spectrum which can be described by a spin Hamiltonian of the form:

$$\mathscr{H} = \beta S\, g\, H + S\, A_{\alpha}\, I_{\alpha} + S\, A_{\beta_1}\, I_{\beta_1} + S\, A_{\beta_2}\, I_{\beta_2}$$

where H is the magnetic field, β is the Bohr magneton and S and I are the electron and nuclear spin operators, respectively. Furthermore, g is the spectroscopic splitting tensor and A_{α}, A_{β_1}, and A_{β_2} are the α and two β-proton hyperfine tensors.

Considerable information has been collected about α and β-proton hyperfine splitting. Thus, A_{α} is quite anisotropic which in the case of powder samples will cause considerable line broadening. The isotropic value is very often of the order 20 gauss. The β-proton splitting is usually isotropic to within 4 gauss. However, the absolute value may vary from zero up to about 60 gauss. This variation is usually ascribed to structural differences in the radical and given by the empirical relation

$$A_{\beta} = (B_0 + B_1 \cos^2 \theta)\varrho$$

where B_0 and B_1 are constants and ϱ is the α-carbon spin density.[18,19] The structural parameter is θ which is the dihedral angle between the direction of the $2p_z$-orbital containing the unpaired spin density and the plane through $C_{\alpha} - C_{\beta} - H$.

With regard to the observed resonance in Fig. 2 A, which consists of 5 resolved lines, the peculiar differences in line width should be pointed out. Thus, the widths (between maximum slopes) from the low field side, given in gauss, are 10.4, 7.6, 12.4, 8 and 10.4. Two different radical models can be proposed which seem to be compatible with these observations:

(1) One of the two radicals I and II are formed. The splitting constants are given in Table 1 and the resulting stick spectrum is given in Fig. 2 B. Whereas the line width for the middle line can be explained by two overlapping lines, it is somewhat harder to understand the differences in linewidth for the two outermost lines on each side. The main factor for the observed line broadening is probably the anisotropic part of the α-proton splitting. Whether the differences in line width can be ascribed to this effect is not known.

Table 1. Hyperfine splitting constants for radicals in uracil.

Radical model [a]	A_{α}	A_{β_1} (in gauss)	A_{β_2}
1. Radical I and/or II	20.5	20.5	48
2. Radical I	22	22	48
Radical II	19.5	19.5	48

[a] According to model 1, only one radical is induced. If the two radicals proposed above have identical hyperfine splitting constants this would also be compatible with model 1. In model 2 two radicals with different splitting constants are proposed.

(2) Both radicals I and II exist. The radicals have different splitting constants and the values given in Table 1 and realized by the stick spectrum in Fig. 2 C will adequately describe the observed resonance including the line width.

In an attempt to decide which of the two alternative models are the most plausible in the present experiments we can obtain information from two sources. In the first place, Herak [20] recently published some data for an X-irradiated single crystal of dihydrouracil. Only one radical was proposed and the observed splitting constants were $A_\alpha = 19.5$ G, $A_{\beta_1} = 24$ G, and $A_{\beta_2} = 42$ G which are in reasonably good agreement with those found in the present experiments. From the direction cosines of the A_α-tensor strong evidence was given in favor of radical I.

The second source of information is theoretical calculations. Thus, Pullman and Mantione,[21] using the Hückel method, claim from energy considerations, that hydrogen addition to C_5 in uracil (yielding radical I) is more favorable than H addition to C_6. Consequently, the calculations and observations previously published lend support to the model that only radical I is formed in dihydrouracil by ionizing radiation.

In the present work the theoretical calculations were extended and the INDO-open shell method was applied. The more interesting results can be summarized briefly as follows:

(1) The energy data slightly favor radical I, but in no way eliminate the existence of radical II.

(2) The isotropic part of the α-proton splitting tensor was smaller than the experimental value throughout. However, the α-proton splitting for radical I was in all calculations 3 to 4 gauss larger than that for radical II. It should be pointed out that this result would support the second model suggested above.

(3) The β-proton splitting obtained in these calculations depends upon the dihedral angle. Even though no definite angular relationship can be given the calculations seem to support the empirical relation given above. Furthermore, it should be noted that the β-proton splitting seems to be very sensitive towards the $C_\beta - H$ bond length and increased with increasing bond distance.

(4) The calculations showed that the $N_1 - H$ and $N_3 - H$ groups share a small part of the unpaired spin density which may result in hyperfine splitting constants of the order 1 to 2 gauss. This interaction will, however, be completely masked in these experiments with powder samples.

In conclusion, the present experiments suggest that when uracil is exposed to thermal H atoms two radicals (marked I and II above) are formed with slightly different splitting constants. This model which adequately explains the observed variation in the line widths is supported by theoretical calculations based on the INDO-open shell method. It should be borne in mind that the single crystal studies of dihydrouracil exposed to ionizing radiation showed another result.

Dose-effect curves. In Fig. 3 are given the dose-effect curves for radical formation in uracil when exposed to the discharge products in a hydrogen gas discharge. The gas pressure was kept at 0.35 torr in these experiments. The dose is given as the exposure time and the different curves refer to powder
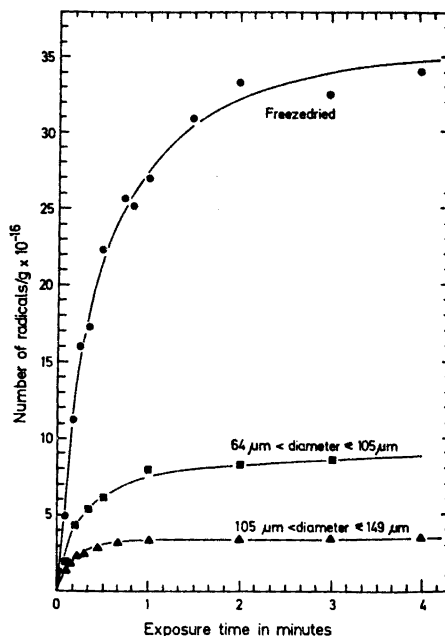
Fig. 3. Dose-effect curves for uracil exposed to hydrogen atoms at room temperature. The dose is given in exposure time assuming that the dose-rate is constant. The different curves refer to uracil particles with different dimensions as obtained by a set of standard sieves.

samples with particle dimensions as given in the figure. Two observations clearly emerged from Fig. 3.

(1) The curves exhibit a clear saturation effect already after a few seconds of exposure. This effect depends slightly upon the particle dimensions, being most pronounced and complete for the largest particles. It should be noted that similar dose-effect curves were obtained also for dihydrouracil.

(2) The yield of radicals, as measured by the initial slope, as well as the saturation level depend strongly upon the particle dimensions. Thus, as shown in Fig. 3 the relative constant plateau reached for freezedried uracil is almost a factor of 10 larger than that for uracil particles with overall dimensions in the range 105 o 150 μ.

The reason for the levelling off of the dose-effect curves seems to be that only a surface layer of the particles is affected by the reactive H atoms. Experiments on thymine seem to indicate that the diffusion length of the H atoms into the particles is of the order 2 to 3 μ.[9] It should be mentioned that after saturation of the surface layer further exposure seems to give rise to radicals deeper into the crystallites. Thus, after the rapid increase of the dose-effect curves the so-called plateau is not completely flat, but the curves show a small increase upon continuous exposure. Whether this is due to thermal hydrogen atoms or to some other discharge product is not known.

Based on the assumption that the induced radicals are confined to a surface layer only, it is possible to arrive at an estimate of the particle dimensions for the freezedried powders. Thus, the results in Fig. 3, assuming spherical particles, yield an average radius of about 10 μ for the freezedried powder.

Effect of the exposure temperature. The above experiments were carried out at room temperature. As described previously it is possible to change the temperature of the sample. This implies that the sample itself can be kept at any desired temperature during exposure. In these experiments uracil and dihydrouracil were exposed at different temperatures and then immediately after exposure transferred to liquid nitrogen temperature and measured without any warming. It should be pointed out that no spectral changes were observed indicating that the same radical is formed at all temperatures. In experiments of this kind it is essential to keep the dose low enough to prevent saturation effects. As shown in Fig. 3 this implies exposure doses of only a few seconds. The variation in radical yield *versus* the exposure temperature is
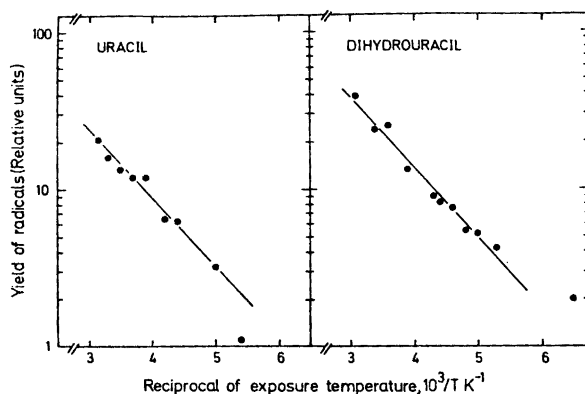

Fig. 4. Yield of radicals in uracil and dihydrouracil as a function of the exposure temperature.

given in Fig. 4. It appears clearly that the yield decreases rapidly with decreasing temperature. The yield data can be adequately fitted by the following reaction:

$$Y(T) = Y_0 \exp(-k/T)$$

One possible interpretation of these results is to assume that the efficiency of the reaction between hydrogen atoms and uracil (dihydrouracil) depends upon the exposure temperature in a similar fashion, *i.e.* the reaction coefficient follows a straight line in an Arrhenius plot. The slope of the straight line would then yield the activation energy for the process responsible for the radical production ($k = \Delta E/R$, where R is the gas constant). From the data in Fig. 4 it can easily be calculated that the activation energy for radical formation by hydrogen atoms in both uracil and dihydrouracil is 2.0 kcal/mol. This value refers to freeze-dried samples. In another experiment with polycrystalline uracil a slightly smaller value of 1.5 kcal/mol was found. These results as well as some previous results for thymine [11] all yield approximately the same activation energy, which might imply that the rate determining step in the formation of radicals is due to one common process. It is possible that the

observed activation energies simply reflect the diffusion of reactive hydrogen atoms into the crystallites rather than the radical forming process itself. This suggestion seems to be supported by the observation that the activation energy is slightly different for polycrystalline uracil.

In Fig. 4 results are given for experiments at temperatures down to about 150 K. By extrapolation of the observed curves down to temperatures like that of liquid nitrogen it can easily be calculated that the yield would be a factor of 10^3 to 10^4 lower than that at room temperature and consequently below the detectability of the spectrometer. In spite of this prediction it appears that prolonged exposure at 77 K (several minutes compared to 20 sec in the experiments shown in Fig. 4, i.e. an increase in dose by a factor of only 10 to 20) yielded detectable amounts of radicals. It has also been reported from other laboratories that radicals are formed when samples kept at liquid nitrogen temperature are exposed to the products in a hydrogen gas discharge system.[8,22] The possibility should be considered that the formation of radicals at 77 K is due to some other discharge products such as UV-light, excited hydrogen molecules or to energetic or hot hydrogen atoms. Support for this suggestion is apparent in Fig. 3 where prolonged bombardment resulted in additional radicals after the first saturation level was reached.

DISCUSSION

The main purpose of using a hydrogen gas discharge system is to create possibilities for a detailed study of the effects of hydrogen atoms. The present and previous studies seem to demonstrate that a certain amount of information can be reached, but that a number of poorly defined parameters such as the gas pressure, the shape of the discharge tube, the exposure temperature, etc., significantly influence the results. Thus, as shown in the present experiments the radical yield versus the gas pressure exhibits an unpredictable relationship. Furthermore, the other products in the gas discharge such as UV-light and excited molecules are not properly defined or controlled. These factors make it difficult to draw any definite conclusions about the reactions between thermal hydrogen atoms and compounds of biological interest and consequently about the efficiency of the method.

As mentioned above, X-irradiated dihydrouracil exhibits a resonance spectrum quite similar to that shown in Fig. 2. Since a variation in the line width was noticed also in those experiments, it is reasonable to suggest that the model involving both radicals I and II is valid. On the other hand, Herak,[20] using the powerful single crystal technique, claims that only radical I is formed by ^{60}Co γ-irradiation.

X-Irradiated uracil yields a more complicated spectrum, which indicates that several radical species are formed. The identification of these radicals would presumably include single crystal studies. The detailed mechanism for the formation of radical I and/or II is not known when ionizing radiation is used. The main problem is to find the source for the hydrogen atoms that are added to the $5-6$ double bond.

Herak and Gordy[4] have previously published some results for uracil bombarded with hydrogen atoms. It is of interest to note that they found

equivalent splitting for the two β-protons in radical I. This is in contrast with the present results, as well as with the single crystal data,[20] which show that one of the β-protons has a splitting almost identical to the isotropic part of the α-proton splitting and only about half the value for the other β-proton. This inequivalence in β-proton splitting can be ascribed to different dihedral angles as mentioned above.

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