Formation of H-Addition Radicals in Adenine Derivatives

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The formation of H-addition radicals in monocrystals of 9-methyl adenine, deoxyadenosine monohydrate, and adenosine hydrochloride by irradiation with X-rays has been studied using ESR spectroscopy. In 9-methyl adenine and adenosine·HCl, hydrogen atoms add exclusively to position C8 of the imidazole part of the purine ring. On the other hand, in deoxyadenosine·H_2O crystals, H-addition radicals at position C2 of the pyrimidine part of the purine ring occur together with H-addition radicals at position C8. Both radicals could be isolated by using their differential stability under warming or illumination with light. The C8-addition radical is characterized by two equivalent β-protons of 38.0 ± 0.5 G and the C2-addition radical by two non-equivalent β-protons of 34.0 ± 1.0 and 50.0 ± 1.0 G. The nitrogen splittings perpendicular to the purine ring are 27.0 and 6.6 G for the C2-addition radical and 20.5 and 9.2 G for the C2-addition radical. The coupling constants of both radicals are in agreement with INDO calculations. It is further shown that the added hydrogen atom comes partly from the hydrogen bonding scheme and partly from the non-exchangeable hydrogens for the C8-addition radical. Together with additional experiments on polycrystalline samples, these results lead to the conclusion that hydrogen atoms add non-selectively to C2 and C8 of the neutral molecules, whereas protons add predominantly to C8 of anion radicals. This is supported by Hückel molecular orbital calculations.

1. Introduction

The formation of H-addition radicals in adenine derivatives has been the subject of previous investigations [1-3]. However, the site of addition remained uncertain. In this work, we present single crystal analysis of the H-addition radicals produced after X-irradiation at room temperature in 9-methyl adenine, deoxyadenosine monohydrate, and adenosine hydrochloride. In the first publications analysing the H-addition radical in single crystals of deoxyadenosine. H_2O different assignments were given. Addition at C8 was proposed by Dertinger [2] whereas Lichter and Gordy [1] concluded that the addition occurs at C2. Later work on polycrystalline samples by Schmidt and Borg [3] showed that both H-addition radicals are simultaneously present. We confirm this conclusion by separation of both radicals utilizing their different stability under warming or illumination with light. It is shown that the methylene proton coupling constants of the C2-addition radical are larger than those of the C8-addition radical and are strongly non-equivalent.

In irradiated 9-methyl adenine a triplet was also detected and assigned to hydrogen addition at C2 or C8 of the purine ring [4]. From studies on polycrystalline samples, Schmidt and Borg [5] deduced

that H-addition occurs exclusively at C8 in this case. We consider 9-methyl adenine as a good model substance for the more complicated adenine derivatives. We have therefore particularly analysed this compound and especially the origins of the hydrogen atoms adding to the purine base. We describe also results obtained after irradiation at 77 K together with those after UV-irradiation at 300 K.

In previous work [6-8], it has been shown that, in pyrimidines with unsubstituted C5 — C6 double bond, addition occurs preferentially at C5 in neutral molecules and at C6 in anionic molecules. Since both C2- and C8-addition radicals have been found in deoxyadenosine·H_2O crystals, we wanted to know if a similar difference between neutral and ionic molecules is also valid for C2 and C8 in adenine derivatives. In order to test this, we used adenosine·HCl crystals. In these crystals, the adenine molecule is protonated at N1 and therefore is a good trap for the secondary electrons produced by X-rays. This should favour an anionic stage in the reaction mechanism.

2. Experimental

9-Methyl adenine was prepared from adenine according to the synthesis of Myers and Zeleznick [9]. The identity of the resulting compound was established by comparison with the NMR spectrum of a solution of 9-methyl adenine obtained from Cyclo

Chemical Corporation. The protons bonded to the amino group were exchanged with deuterons by warming a D_2O solution of 9-methyl adenine at $40-50\,°C$ for $2-3$ hours. In addition, the proton bonded to C8 could be exchanged by warming a D_2O solution for 8 to 12 hours at $110\,°C$. Crystals could thus be obtained fully protonated (A), with the amino group deuterated (B), with the amino group and the proton at the C8 carbon deuterated (C), or with only the proton at the C8 carbon replaced by a deuteron (D). Single crystals were grown by slow evaporation at room temperature of solutions saturated at $30-40\,°C$ in a desiccator. The molecular structure has been reported by Stewart and Jensen [10]. The crystals are monoclinic with space group $P2_1/c$. There are four molecules in the unit cell. For the ESR measurements, an orthogonal coordinate system a^*, b, c was chosen. The molecules are coplanar and the plane of the purine rings is roughly parallel to the (b,c)-plane.

Single crystals of deoxyadenosine were grown by slow evaporation at room temperature of aqueous solutions. The crystals are monoclinic with space group $P2_1$ [11]. There are two molecules per unit cell. The same orthogonal system a^*, b, c as that of Lichter and Gordy [1] was chosen for the ESR measurements. The normals of the purine rings make an angle of about $16°$ with the b-axis. The proton bonded to C8 was exchanged for deuterium by warming a D_2O solution of deoxyadenosine for 14 hours at $70-80\,°C$. The crystal types A, B, C, D equivalent to those of 9-methyl adenine were also grown with deoxyadenosine. Single crystals of adenosine·HCl were grown from 1 N HCl solutions at $4\,°C$. The crystals are monoclinic with space group $P2_1$ and two molecules per unit cell [12].

The single crystals were irradiated with 100 kV X-rays at doses ranging from 0.1 to 10.0 Mrad. Illumination with UV light was done with a 1000 W HBO Osram lamp. The ESR spectra were registered as first derivatives with a Varian E-9 spectrometer at 9.5 GHz and at 35 GHz. Thec rystals were rotated within three mutually perpendicular planes of the (a^*, b, c)-frame in steps of $6°$ using an automatic goniometer described elsewhere [13]. Computer simulations of spectra were carried out using a Varian 620i computer interfaced with the ESR spectrometer and a simulation programme supplied by Varian.
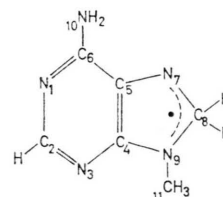
3. 9-Methyl Adenine

3.1. *The hydrogen addition position*

After X-irradiation at 300 K, radicals **1** and **2** are produced [4]. In order to analyse radical **2** without
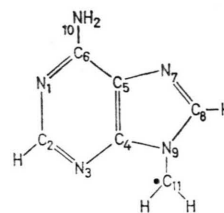
Radical 1

Radical 2

the overlapping lines of radical **1**, several treatments were attempted for separating the superimposed spectra. This could only be attained by illuminating with UV-light the X-irradiated crystal at 300 K. In this way, radical **1** was totally photobleached leaving radical **2** unaltered. However, a singlet component appeared in the middle of the spectrum. In Fig. 1, the spectra obtained with the magnetic field

Fig. 1. ESR spectra of a single crystal of 9-methyl adenine X-irradiated at 300 K. The magnetic field is parallel to the *c*-axis. Top spectrum: immediately after X-irradiation, the stick diagrams indicates the lines due to the abstraction radical (radical **1**). Bottom spectrum: after illumination with UV-light at 300 K, the stick diagram indicates the lines due to the addition radical (radical **2**). See 3.1 for the additional triplet splitting present in the abstraction radical spectrum.

parallel to the c-axis before and after irradiation with UV-light are shown. The hyperfine structure present in the lines due to radical **1** originates from the interaction of the unpaired electron with nitrogen N9. A similar procedure has been used previously for separating the addition radical from the abstraction radical produced in irradiated single crystals of 9-ethyl adenine [14].

The spectrum of radical **2** is a 1:2:1 triplet of splitting around 38.0 G. The splitting is isotropic, which indicates interaction of the unpaired electron with two equivalent β-protons. Further, when the magnetic field is oriented perpendicular to the purine ring, several lines appear (Fig. 2). These lines are
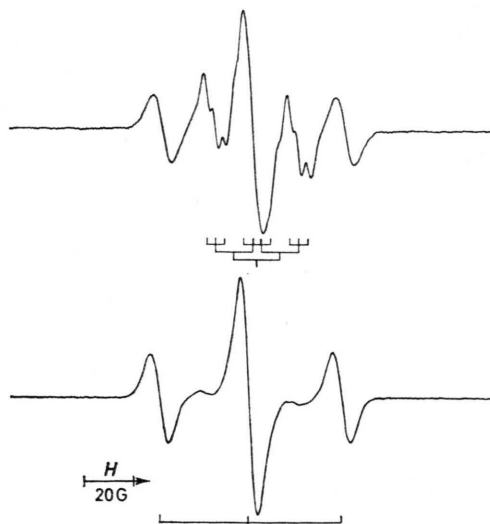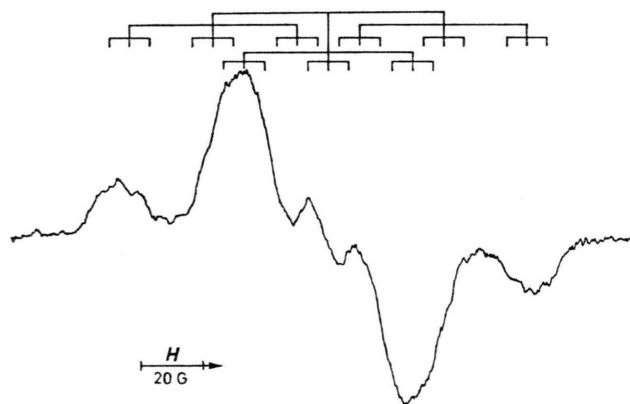

Fig. 2. ESR spectrum of a single crystal of 9-methyl adenine X-irradiated at 300 K. The magnetic field is parallel to the a^*-axis, i. e. roughly perpendicular to the purine rings. The stick diagram indicates the couplings due to two equivalent methylene protons and to two non-equivalent nitrogens.

typical of interaction with two inequivalent nitrogen nuclei. At this orientation, the couplings are as following:

$$A(H_\beta) = 38.0 \pm 0.5 \text{ G}$$
$$A(N') = 27.0 \pm 0.5 \text{ G}$$
$$A(N'') = 6.6 \pm 0.5 \text{ G}.$$

These values are identical to those found in a single crystal of 9-ethyl adenine for a radical attributed to the same ESR patterns [14]. After comparison between experimental and calculated couplings obtained with the INDO method, it was concluded that the H-addition occurs at C8. The similarity of the values in both crystals shows that the position of the H-addition is the same in 9-methyl adenine and in 9-ethyl adenine. Another observation supports this conclusion. Irradiation of single

crystals of type (D) yields spectra typical of HD-addition radicals: the triplet pattern is replaced by a double pattern.

It should be mentioned that polycrystalline samples of adenine also present a 1:2:1 triplet of 38.0 G splitting after irradiation at room temperature which collapses into a singlet if all protons bonded to the nitrogen atoms and to the carbon C8 are carefully removed by deuteration. This shows that the hydrogen atom adds exclusively to position C8 in pure adenine and in 9-methyl adenine. A similar conclusion was reached after analysis of single crystals of adenine hydrochlorides [15]. These results are contradictory to those of Schmidt and Borg [5] who claimed that H-addition at position C2 also occurs in adenine free base and adenine hydrochloride.

3.2. The origin of the added hydrogen atom

Four types of single crystals A, B, C, D, with different positions deuterated were quantitatively analysed in the following way. The crystals were X-irradiated at the same doses and then the abstraction radical was photobleached leaving a singlet spectrum and the addition radicals consisting of H- or D-addition radicals in various proportions. From the knowledge of the ESR parameters, it is possible to simulate the spectrum parallel to the c-axis for the HH-addition radical to C8. The HD- or DD-addition radicals could also be simulated by dividing the β-proton coupling by 6.51. The spectra of the A, B, C, D crystals were then simulated by adding the various H-addition radicals in proportions giving best agreement with the experimental spectra. The experimental and simulated spectra are presented in Fig. 3. The agreement is seen to be excellent. The proportions of the different radicals are contained in Table I. The concentration of the singlet pattern increases from the A to the D type

Table I. Relative proportions of addition radicals and of the singlet pattern in the four crystals A, B, C, D as determined by simulation of the experimental spectra (see Fig. 3). The proportions of the various addition radicals are also given.

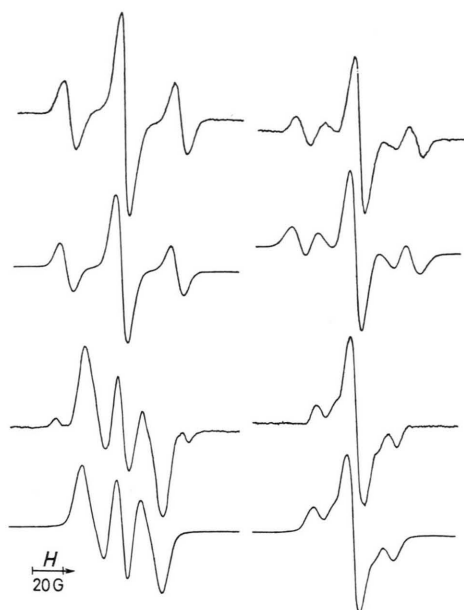| Crystal	Singlet	Addition	HH	HD	DD
A	35	65	100		
B	45	55	60	40	
C	50	50		40	60
D	45	55	100		

Fig. 3. Experimental (upper) and simulated (lower) spectra of lingle crystals A (top left), B (top right), C (bottom right), and D (bottom left). X-irradiated at 300 K with the same dose. The magnetic field is parallel to the *c*-axis. The satellite lines present in the experimental spectrum of crystal (D) are assigned to HH-addition radicals which occur because of a slight back-exchange deuteron-proton at C8.
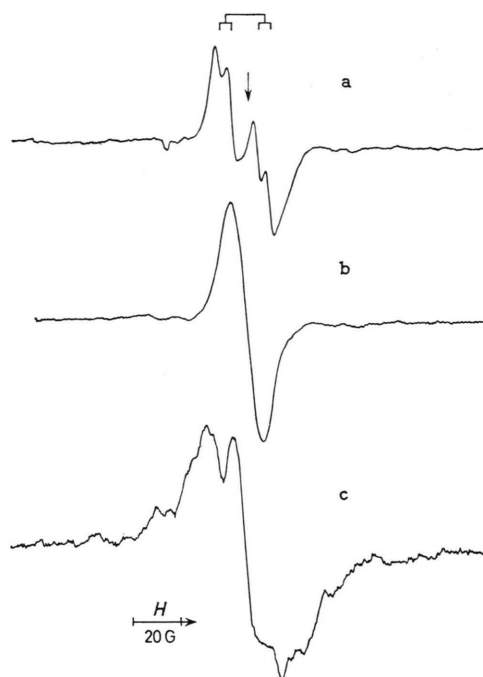

Fig. 4. ESR spectra of a single crystal of 9-methyl adenine X-irradiated at 77 K: the magnetic field is parallel to a. the *b*-axis, b. the *c*-axis, c. the *a**-axis. The arrow indicates the position of DPPH.

crystals. This singlet spectrum is observed after photobleaching of the X-irradiated crystals and could therefore be a degradation product of the abstraction radical.

The presence of HH-radicals in the B-crystals and of HD-radicals in the C-crystals definitively proves that part of the added hydrogen atoms originate from the methyl group. Furthermore, the presence of HD-radicals in B-crystals and of DD-radicals in C-crystals shows that the rest of the added hydrogen atoms comes from the hydrogen bonding network. Considering the errors involved in such procedures, it can be concluded that about 50% of the added hydrogen atoms originate from the hydrogen bonds with the other 50% coming from the methyl group.

3.3. *The occurence of the C8-addition radical*

After irradiation at 77 K, another radical characterized by a broad singlet, with supplementary couplings at some orientations, is produced (Fig. 4). The lines of the addition and of the abstraction radicals can also be detected at high gain. Upon irradiation with red light or annealing to

150 K, this low-temperature radical disappears and the lines of the other two radicals are better resolved. The yields of radicals **1** and **2** are between 2 and 3 orders of magnitudes lower after irradiation at 77 K and warming to 300 K than immediately after irradiation at 300 K. It has not been possible to observe transformation of the low-temperature radical into radical **1** or radical **2**.

HH-addition radicals as well as HD-addition radicals are observed in irradiated B-crystals at 77 K. However, in C- and D-crystals, no HH-addition radicals are detected. Therefore, the conclusions reached from the results of X-irradiation at 300 K also apply to the observations made after X-irradiation at 77 K. UV-irradiation of non X-irradiated single crystals of 9-methyl adenine at 77 K and at 300 K produces addition radicals. Abstraction radicals were only produced after UV-irradiation at 77 K. The analysis of the spectra of crystals A, B, C, D shows that the position of addition is still C8 and that the hydrogen atoms are partly coming from the methyl group and partly from the hydrogen bonds. To our knowledge, it is the first report on the production of free radicals in a nucleic acid constituent single crystal by UV-light. Several

pyrimidine crystals have been UV-irradiated at 77 K and at 300 K without giving any detectable signal.

4. Deoxyadenosine Monohydrate

By using computer techniques which sharpen the lines of an ESR spectrum, Schmidt and Borg [5] concluded that in deoxyadenosine both types of H-addition radicals occur. However, they could only state that the spectrum of one was larger than the other. We could achieve complete isolation of the spectra of both radicals by warming or illuminating with light the crystals subsequently to X-irradiation.

In Fig. 5, the effect of warming for 20 to 30 min at 100 °C a crystal of deoxyadenosine X-irradiated
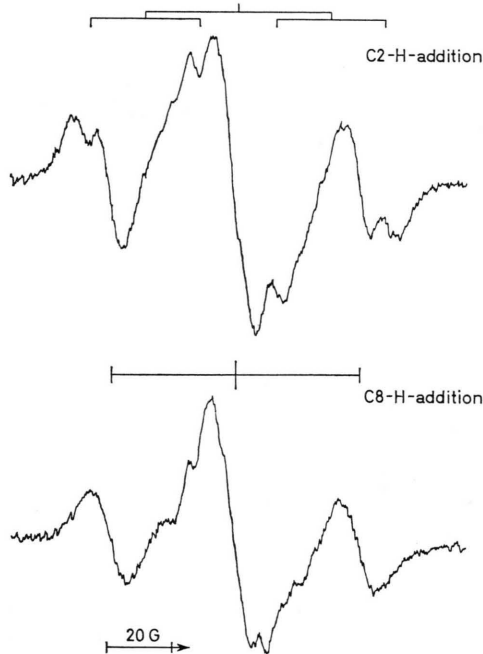

Fig. 5. ESR spectra of a single crystal of deoxyadenosine. H_2O X-irradiated at 300 K. Top spectrum: immediately after X-irradiation; the stick diagram indicate both H-addition radicals. Bottom spectrum: after warming the crystal for 20 min at 100 °C; only the lines of the C8-addition can be still observed. In Figs 5—7, the additional lines present in the middle are due to a sugar radical [1, 19].

at room temperature is shown. In the upper spectrum the extreme lines present structure, which disappears after warming the crystal (lower spectrum). The lower spectrum is mainly at 1:2:1 triplet with 38.0 ± 0.5 G splitting. We assign this triplet to the C8-addition radical. Indeed, it can be seen in
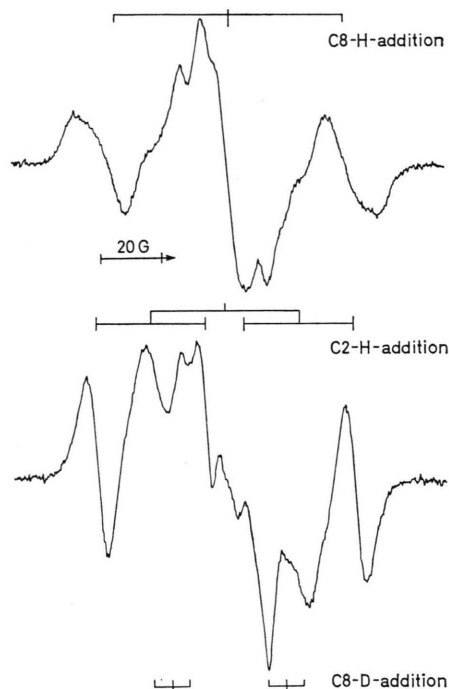

Fig. 6. Top spectrum: ESR spectrum of a single crystal of deoxyadenosine. H_2O X-irradiated at 300 K; both H-addition radicals are present. Lower spectrum: same conditions, but the proton bonded to C8 has been exchanged for a deuteron; the H-addition radical at C2 and the D-addition radical at C8 are indicated by the stick diagrams. The magnetic field is at 55° from the c-axis in the (a^*, c)-plane.

Fig. 6 that replacement of the C8 proton by a deuteron leads to new lines near the middle of the spectrum. These lines are typical of a D-addition radical. Furthermore, the methylene proton coupling constants are identical with those observed in 9-methyl adenine (see 3.1) and in 9-ethyl adenine [14].
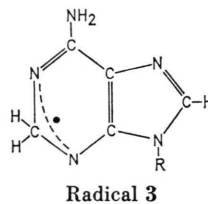
Radical **3**

On the other hand, Fig. 7 shows the result of illuminating with light of $\lambda > 360$ nm a crystal X-irradiated at room temperature. The lines assigned to the C8-addition radical disappear, leaving a 1:1:1:1 quartet. We attribute these lines to the C2-addition radical with unequal β-proton coupling constants (radical **3**). The two methylene proton
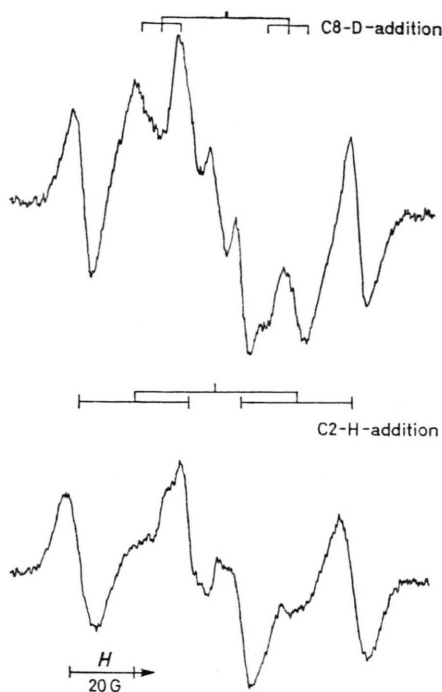
Fig. 7. ESR spectra of a single crystal of deoxyadenosine·H_2O with the C8 proton replaced by a deuteron and X-irradiated at 300 K. Top spectrum: immediately after X-irradiation; H-addition at C2 and D-addition at C8 are present. Lower spectrum: after illumination with light of $\lambda > 360$ nm at 300 K for 3 hours; only the lines of the C2-addition radical can still be observed. Same orientation as in Fig. 6.

Table II. Theoretical and experimental couplings for both H-addition radicals of adenine. The theoretical values are taken from reference [14]. For the nitrogens, only the values obtained with the magnetic field parallel to the $2p_z$-orbital are given. All values are in gauss. For the C2-addition radical the mean value of the methylene proton coupling constants is given.

Radical	H (β)	N'	N''
C8-addition N' = N7 N'' = N9	42.2	27.0	6.4
Experimental	∼38.0	27.0	6.6
C2-addition N' = N3 N'' = N1	50.2	25.1	11.0
Experimental	∼42.0	20.7	9.2

coupling constants are respectively 34.0 ± 1.0 G and 50.0 ± 1.0 G. Their mean value is therefore larger by about 4.0 G than that of the methylene proton coupling constants of the C8-addition radical. Since the non-equivalence of the methylene protons has not been reported by Lichter and Gordy [1], we remeasured the nitrogen couplings. The results are contained in Table II together with those for the C8-addition radical. In Table II, are also presented the results of INDO calculations on both radicals which were made previously [14]. The overall agreement between experimental and theoretical couplings is very good. It can be noted that the increase in the methylene proton couplings is well reproduced. The small nitrogen coupling is also calculated larger in the C2-addition than in the C8-addition radical in accord with experience.

From their study on polycrystalline samples, Schmidt and Borg [5] inferred that the hydrogen atoms adding to C2 originate exclusively from the easily exchangeable positions. However, in crystals

grown from D_2O (type B), we could observe H-addition to C2. Futher, in crystals with the C8 proton exchanged for a deuteron and grown from D_2O (type C), H-addition radicals at C8 could be observed. This indicates that exchangeable as well as non-exchangeable hydrogens add to position C8 and that non-exchangeable hydrogens add to position C2. We could not prove the occurence of addition of easily exchangeable hydrogens to position C2.

5. Discussion and Conclusions

The single crystal analysis presented in this paper corroborates some of the conclusions made by Schmidt and Borg [5] on the basis of polycrystalline studies. The preferred site of attack is the carbon C8 of the imidazole part of the purine ring. However, attack on the carbon C2 of the pyrimidine part of the purine ring also occurs depending on the molecular environment. Irradiation of aqueous solutions of adenine also leads to the conclusion that the predominant site of attack is the carbon C8 [16, 17]. Hückel molecular orbital calculations show that attack by free radical occurs on C8 rather than on C2 in the neutral molecule [18]. The distinction between C8 and C2 is not very strong. Thus, hydrogen addition should occur on C8 or C2 in the neutral molecules depending on the local availability of hydrogen.

The preference for C8 is, however, increased over that for C2 in case of protonation of the anion radical of the adenine molecule [6]. This could explain that, in the hydrochloride salts of adenine,

only the C8-addition radicals were detected [15]. Hüttermann [15] also postulates the existence of an anionic stage for the C8-addition radicals. In these crystals, the adenine base is protonated and can therefore act as a better electron acceptor than the neutral base. To see if this mechanism would still be valid in case of nucleosides, we studied single crystals of adenosine·HCl *. Only the C8-addition radicals characterized by a triplet of 38.0 G splitting were observed. However, powder spectra of pure adenosine indicate that C2- and C8-addition radicals are present. Therefore, as in pyrimidines, the site of hydrogenation depends on the state of the molecule. Protons seem to react exclusively with the C8 position of the electron adduct of the adenine base and hydrogen atoms non-selectively with C2 or C8 of the neutral adenine base.

* *Note added in proof:* Further investigations indicate that C2-addition radicals are also present in X-irradiated adenosine·HCl crystals. However, they have the same methylene couplings as the C8-addition radicals. INDO

The study of single crystals containing selectively deuterated molecules showed that part of the added hydrogen atoms were abstracted from the N9-substituent (methyl group or sugar) with the rest coming from the hydrogen bonding network (the easily exchangeable hydrogens). Radicals resulting from hydrogen abstraction from the methyl group or from the sugar were identified in 9-methyl adenine and in deoxyadenosine [4, 19]. Radicals resulting from rupture of the hydrogen bonding network were not identified. In 9-methyl adenine crystals, the non-identified species formed after X-irradiation at 77 K could be related to the damage done to the hydrogen bonds.

calculations show that, when the purine base is protonated at N1, the methylene couplings of both H-addition radicals are identical. These results will be published later in full detail.

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