Site of Protonation of One-Electron-Reduced Cytosine and Its Derivatives in Aqueous Methanol Glasses

Paul M. Cullis,* Mark E. Malone, Ian D. Podmore, and Martyn C. R. Symons†

Department of Chemistry, Leicester University, Leicester LE1 7RH, U.K.

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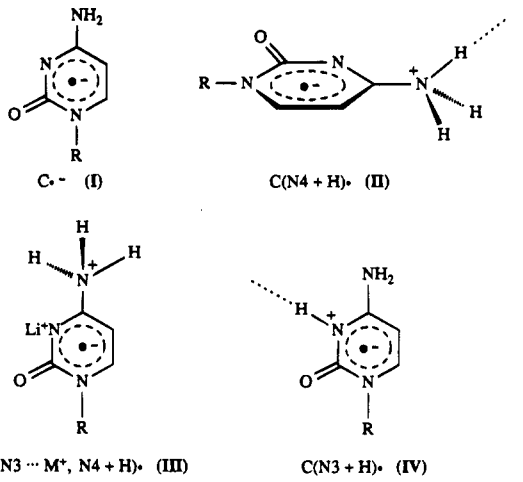
Electron paramagnetic resonance spectra of one-electron-reduced cytosine, cytidine, 2′-deoxycytidine, 5′-dCMP, and 3′-dCMP produced by ^{60}Co γ-irradiation in aqueous lithium chloride glasses ($LiCl/H_2O$) and aqueous methanol glasses ($MeOH/H_2O$) at 77 K exhibited a triplet which has been interpreted in terms of the radical anion protonating on the exocyclic amino group of the base C(N4+H)• (**II**). The exchangeable nature of the extra proton coupling is demonstrated by the collapse of the triplet into a doublet in $LiCl/D_2O$ and CD_3OD/D_2O glasses. The EPR spectrum of cytidine-*5,6-d*$_2$ in either LiCl glasses or aqueous methanol led to a doublet in each case, consistent with the loss of the coupling to the hydrogen on C6, with the remaining coupling due to the protonation on the exocyclic amino group. Comparable experiments in $LiCl/D_2O$ or CD_3OD/D_2O glasses led directly to a singlet. The results in aqueous methanol glasses parallel closely those in aqueous LiCl glasses and provide clear evidence for protonation on the exocyclic amino group even in the absence of metal ion coordination to N3. Hence, N4 protonation of the cytosine radical anion is not a result of metal ion coordination to N3 as has been suggested by others. We suggest that N4 protonation is kinetically controlled, possibly brought about by solvation effects, whereas the N3 protonation would occur under thermodynamic control.

Introduction

The direct ionization of DNA is widely presumed to make a significant contribution to damage to nuclear DNA *in vivo*. EPR spectroscopy of aqueous DNA irradiated at low temperatures has been used to explore the mechanism of the direct damage pathway and has established that direct ionization of DNA leads to two major radical species.[1-6] The one-electron loss center has been assigned to the radical cation of guanine ($G^{\bullet+}$); however, the one-electron gain center has proved rather more controversial. Although there is agreement that the radical anion centers are confined to the pyrimidine bases, detailed assignment has proved difficult. Initially the poorly resolved anisotropic doublet with *ca.* 1.6 mT splitting was assigned to the radical anion of thymine ($T^{\bullet-}$), in part because of the significant yields of the 5,6-dihydrothymin-5-yl radical ($TH^{\bullet}$) on annealing.[2-6] However, more recent evidence has been presented that the doublet arises at least in part from the one-electron adduct of cytosine.[7-12] This assignment has been made on the basis of careful simulation of Q-band spectra of oligodeoxynucleotides irradiated in LiCl glasses by Bernhard and co-workers[7-9] and X-band studies on irradiated DNA and polydeoxynucleotides in frozen aqueous and LiCl glasses by Cullis *et al.*[10] and Sevilla *et al.*[11] In addition Hüttermann *et al.*[12] have concluded on the basis of studies on orientated DNA containing thymine deuterated at the methyl group that the major radical anion site is $C^{\bullet-}$ and not $T^{\bullet-}$.

The recognition of the potential importance of $C^{\bullet-}$ (**I**) in the radiolysis of DNA has prompted a number of recent model studies on cytosine and its derivatives. We reported the unexpected observation that irradiation of cytosine and its derivatives in aqueous LiCl glasses at 77 K led to triplet EPR spectra rather than the doublet seen in DNA.[13,14] We attributed



this to the protonation of the exocyclic amino group orthogonal to the plane of the ring C(N4+H)• (**II**), and this has been confirmed and accepted by others.[15-17] This result was surprising, since the preferred site of protonation is expected to be N3 to give C(N3+H)• (**IV**), which is presumed to be what happens in DNA, and it is difficult to account for the switch in the site of protonation seen in monomeric systems.[18,19] It has been suggested that protonation at the exocyclic amino group of $C^{\bullet-}$ in glasses containing alkali metal ions occurs because coordination of metal cations from the glass to N3 blocks protonation at the preferred site C(N3•••M^+,N4+H)• (**III**).[15-17] The amino group protonation would thus be an artifact of the LiCl matrix. At odds with this is our preliminary observation for irradiation of 2′-deoxycytidine in aqueous methanol glasses which also showed a triplet spectrum[14] although this has been called into question by others.[15,16] We report here an extensive study of the one-electron-reduced radicals of cytosine and its derivatives, including the deuterated nucleoside cytidine-*5,6-d*$_2$, in $CH_3OH/$

* Address correspondence to this author: Professor P. M. Cullis, Department of Chemistry, Leicester University, Leicester LE1 7RH, U.K. E-mail: PMC@leicester.ac.uk.

† Present address: Department of Chemistry, The University of Essex, Wivenhoe Park, Colchester, U.K.

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H_2O and CD_3OD/D_2O glasses that provide unambiguous evidence for the protonation of the exocyclic amino group *even in the absence of metal salts.*

Experimental Section

Materials. Cytosine, cytidine, 2′-deoxycytidine (2′dC), cytidine 5′-monophosphate (CMP), 2′-deoxycytidine 5′-monophosphate (5′dCMP), 2′-deoxycytidine 3′-monophosphate (3′dCMP), and *N,N*-dimethyl-2′-deoxycytidine were obtained from Sigma Chemical Company. High-purity LiCl, $K_3Fe(CN)_6$, CD_3OD, D_2O, and DMSO-d_6 were obtained from Aldrich Chemical Co. HPLC-grade methanol was obtained from Fisons. These materials were used without further purification. H_2O was obtained from a millipore "Multi Q" purifier.

5,6-Dideuteriocytidine (cytidine-*5,6-d*$_2$) was synthesized by the method of Rabi and Fox,[20] which is briefly described below. Cytidine (0.48 g) was dissolved in 4.5 mL of DMSO-d_6 and 1.7 mL of 2.5 M CD_3ONa in the CD_3OD, and the solution was heated at 60 °C for *ca.* 18 h. The reaction mixture was neutralized with 1 M HCl and loaded onto a 1:1 (w/w) charcoal/cellulose column and washed with water until the eluent was chloride ion negative ($AgNO_3$ test). The exchanged nucleoside was then eluted from the column with a 50% $EtOH/H_2O$ mobile phase, and the collected fractions were concentrated, redissolved in a small amount of water, and lyophilized to a fine white powder. The ^1H NMR spectrum confirmed that the C5 and C6 positions were deuterated to greater than 95% and the product was fully characterized.

Procedure. Two glasses at neutral pH were employed for this study: 10 M LiCl glasses (H_2O and D_2O) and methanol water glasses (CH_3OH/H_2O and CD_3OD/D_2O) at a ratio of 9:2 (v/v). With the exception of the case for cytosine, glasses were made using solute concentrations of 50 mM. Cytosine was used at a concentration of only 35 mM due to its low solubility. Frozen beads were formed by micropipeting small drops of solution into a reservior of liquid nitrogen.

Samples were γ-irradiated at 77 K using a Vickrad ^{60}Co γ-ray source to a dose of 1 Mrad. EPR spectra were recorded at 77 K on a JEOL JES-RE1X X-band spectrometer, interfaced with an Archimedes computer. Unless otherwise stated a microwave power of 10 μW and a modulation amplitude of 0.2 mT were used for each spectrum. The radical anion spectra in LiCl glasses were obtained directly at 77 K and on annealing to 155 K, at which point the solvent radicals ($Cl_2^{\bullet-}$ and $ClOH^{\bullet-}$) decay. In the aqueous methanol glasses the radical anion spectra were obtained after warming to *ca.* 130 K, thereby allowing the trapped electron to become mobile and removing the masking solvent features from the $^{\bullet}CH_2OH$ radical. To test whether electron adducts do form and that the observed solute spectra are not artifacts of the glassy matrices, a powerful electron scavenger [$K_3Fe(CN)_6$] was employed. Complete suppression of the solute spectra was achieved with 5 mg/mL ferricyanide, thereby indicating that the triplet and doublet spectra observed in these glasses are due to the one-electron cytosine adducts.

EPR Simulations. The hyperfine coupling constants from each of the spectra referred to in the text were obtained from the best-fit simulations which were performed using isotropic **g** and isotropic line width tensors (0.3 mT). The H6 hyperfine coupling was simulated using an anisotropic proton splitting of 1.5, 0.6, and 2.25 mT, as reported by Flossmann *et al.*[21] The best-fit simulation of the triplet was achieved using an additional isotropic coupling of 1.2 mT for the added orthogonal proton at N4 and 0.3 mT for the other two protons. In the deuterated solvents the *ca.* 1.2 mT coupling is reduced to *ca.* 0.2 mT, which is lost in the line width of the residual doublet (Figure 2).

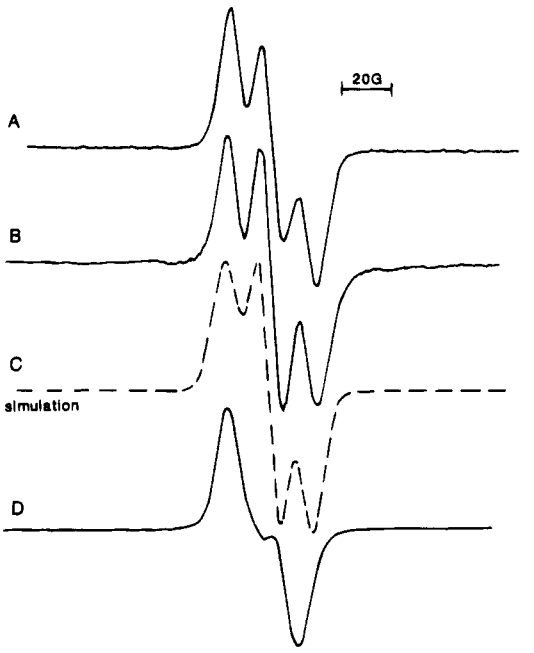

Figure 1. X-Band EPR spectra of 2′-deoxycytidine γ-irradiated at 77 K: (A) In a $MeOH/H_2O$ glass after annealing to 130 K showing the triplet spectrum assigned to the N4-protonated radical anion C(N4+H)$^{\bullet}$; (B) In a $LiCl/H_2O$ glass after annealing to 155 K showing a triplet with spectral parameters very similar to those in the methanol glass; (C) Simulation of (a) and (b) based on the parameters in the text; (D) In the $MeOH/H_2O$ glass after warming to the softening point of the glass, where the triplet collapses to a poorly resolved doublet. All spectra were recorded at 77 K and are shown without any modification.

The C6−D hyperfine coupling for the cytidine-*5,6-d*$_2$ cytidine was simulated using an isotropic coupling of 0.23 mT.

Results

2′-Deoxycytidine. The EPR spectrum of 2-deoxycytidine irradiated at 77 K in aqueous methanol glasses and annealed to 130 K to remove radicals derived from the matrix showed a characteristic triplet with spectral parameters closely similar to those previously reported for the irradiation of 2-deoxycytidine in LiCl glasses (Figure 1A and B, respectively). Further annealing of the $MeOH/H_2O$ sample led to the collapse of the triplet to a somewhat poorly resolved doublet (Figure 1D). This annealing of aqueous methanol glasses is technically quite difficult, since the onset of the collapse of the triplet to the doublet occurs at temperatures that are very close to the glass-softening point. We have previously assigned the triplet to the radical anion $C^{\bullet-}$ that has been protonated on the exocyclic amino group, C(N4+H)$^{\bullet}$ (**II**), and in the case of LiCl and other alkali metal glasses, this has been confirmed subsequently by others. The triplet arises from coupling to the C6 proton and the added proton at the exocyclic amino group which is assumed to be held orthogonal to the plane of the ring by hydrogen bonding in the rigid matrix. Spectral analysis indicates that the spectrum is due to a proton coupling of *ca.* 1.5 mT (C6−H) and an additional exchangeable proton coupling of 1.2 mT. Computer simulation using these parameters with a line width of 0.3 mT gave a good fit to the experimental spectrum (Figure 1C). The collapse of the triplet on annealing the aqueous methanol glasses was previously attributed to the onset of rotation of the NH_3^+ group at temperatures close to the glass-softening point, which corresponds to the point at which the large coupling from the single proton orthogonal to the plane is lost.[14] A range of conformations of the NH_3^+ group were presumed to result and become trapped in the matrix on

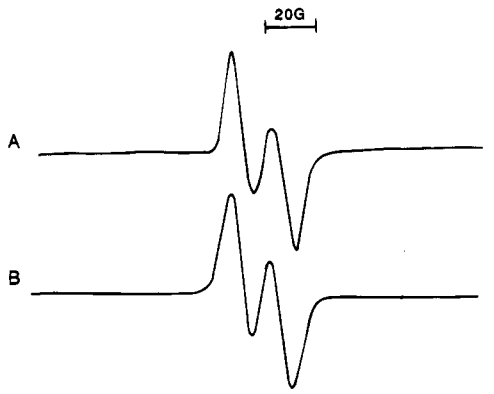

Figure 2. X-Band spectra of (A) γ-irradiated 2′-deoxycytidine at 77 K in CD_3OD/D_2O after annealing to 130 K showing the doublet spectrum assigned to the exchangeable N4 deuteration of the radical anion C(N4+D)• leading to the collapse of the triplet spectrum observed in $MeOH/H_2O$ glasses (Figure 1A) and (B) γ-irradiated *N,N*-dimethyl-2′-deoxycytidine in a $MeOH/H_2O$ glass showing a doublet spectrum due to the absence of orthogonal protonation at N4. All spectra were recorded at 77 K and are shown without any modification.

recooling to 77 K prior to recording the spectrum. If this were the case, the simulation of this spectrum is not trivial, since it will be a superposition of many different spectra arising from the many different conformations of the NH_3^+ group. Our previous simulation[14] showed that a broad doublet could be obtained assuming three equivalent NH_3^+ protons with A_{iso} = 0.58 mT, but this simulation is only hypothetical, since the NH_3^+ group is not freely rotating. Furthermore, the recent work by Bernhard on $C^{\bullet-}$ in acidic glasses[17] reported a quartet EPR spectrum for $C(N3+H,N4+H)^{\bullet+}$ arising from the coupling to the C6−H and two β-couplings from the NH_3^+ in a conformation in which one NH is in the plane of the ring and the remaining two N−H's have reasonably large hyperfine couplings. If this assignment is correct and if this conformation is the lowest energy, as suggested, we might have expected to observe quartet EPR spectra on annealing both the neutral aqueous methanol glasses and neutral aqueous LiCl glasses, neither of which was seen. The alternative explanation for the collapse of the triplet would be that annealing the sample to the glass-softening point allows proton transfer from N4 to N3. This explanation, however, also present difficulties, since the triplet collapses to a doublet that is significantly broader from C(N3+H)• seen in other systems such as poly(dG-dC) and other oligodeoxynucleotides.[7,14] For this alternative explanation to be correct, we must assume that the proton transfer is incomplete in order to account for the broadening of the spectra. Finally, irradiation of 2′-deoxycytidine in CD_3OD/D_2O glasses gave rise to a doublet where the coupling to the ND_3^+ deuterons (*ca.* 0.2 mT) is lost in the line width of the doublet arising from coupling to the C6 proton (Figure 2A), similar to experiments conducted in D_2O/LiCl glasses.

Many of the cytosine derivatives that we have studied in aqueous methanol glasses show similar results to 2′-deoxycytidine, including cytosine, cytidine, 5′-dCMP, and 3′-dCMP. The spectral parameters are summarized in Table 1. For all of these a triplet spectrum is readily obtained in the presence of H_2O with a spectral width similar to that for the C(N4+H)• (**II**) radical produced in $LiCl/H_2O$ glasses. Confirmation that this radical species giving rise to the triplet is derived from a radical anion was obtained by showing that the triplet was not formed when cytidine was irradiated in aqueous methanol glasses in the presence of the electron scavenger $K_3Fe(CN)_6$ and subsequently annealed to 130 K.

***N,N*-Dimethyl-2′-deoxycytidine.** In the case of *N,N*-dimethyl-2′-deoxycytidine in aqueous methanol glasses, a doublet with a spectral width of 2.55 mT is obtained (Figure 2B), which suggests that the site of protonation for this derivative is no longer N4, and we assume that the radical anion obtained from this derivative now protonates on N3. The same switch in site of protonation between 2′-deoxycytidine and *N,N*-dimethyl-2′-deoxycytidine was also observed on irradiation in LiCl glasses.[14,16] We rationalized this in terms of the difficulty associated with pyramidalizing the amino group on N4 protonation of the radical anion of *N,N*-dimethyl-2′-deoxycytidine, which would require significant movement of the bulky methyl groups. This effect must be sufficient to switch the protonation to N3. An alternative explanation would be that the radical anion of *N,N*-dimethyl-2′-deoxycytidine is not protonated at all. However, the fact that cytidine in sodium hydroxide glasses still shows a triplet species (data not shown) suggests that $C^{\bullet-}$ (**I**) must be an extremely strong base. In fact the basicity of $C^{\bullet-}$ is reported to increase by ≥9 orders of magnitude in comparison to that of C.[18] In view of this we consider it unlikely that methylation of the exocyclic nitrogen will shift the pK_a sufficiently to prevent protonation. It is also possible that N4 protonation has taken place but that the added proton is no longer held orthogonal to the plane of the ring. Certainly the NMe_2 group will not be hydrogen bonded in the matrix, as would be the case for the NH_2 group, and this would facilitate rotation of the $NHMe_2^+$, but it seems somewhat unlikely that this would occur at 77 K.

Cytidine-*5,6-d2*. In view of the importance of our observation that N4 protonation of the one-electron-reduced cytosine occurs even in the absence of metal cations, we have conducted studies on deuterated cytidine to confirm the radical assignment. Cytidine-*5,6*-d_2 can be readily prepared by base-induced exchange of the protons in cytidine. The ^1H NMR spectrum confirmed that both the C5 and C6 positions were deuterated to greater than 95% and the product was fully characterized. Irradiation of 5,6-dideuteriocytidine in LiCl glasses gave rise to a poorly resolved doublet (Δ = 2.15 mT) in which the major coupling (1.2 mT) arises from the proton added to N4 and the reduced coupling to deuterium at C6 is not resolved (Figure 3A). Simulation of this spectrum was achieved using a C6 deuterium coupling of *ca.* 0.2 mT together with a 1.2 mT coupling for the added N4 proton and a *ca.* 0.3 mT coupling for the other two amino protons. The corresponding experiment in D_2O/LiCl glasses, as expected, gave rise to a singlet due to the reduced coupling to both C6−D and the orthogonal N4−D (Figure 3C and D).

Irradiation of cytidine-*5,6-d*$_2$ in aqueous methanol glasses similarly gave rise to a poorly resolved doublet (Δ = 1.8 mT) consistent with loss of the coupling to the C6 proton in the C(N4+H)• radical (Figure 4A). Annealing this sample to the softening point of the glass led to the collapse of the doublet to a singlet, as expected on the basis of all of the previous observations (Figure 4B). Finally, irradiation of cytidine-*5,6-d*$_2$ in CD_3OD/D_2O glasses gave rise to a singlet consistent with the loss of the two large couplings to the C6 proton and the orthogonal N4−H (Figure 4C).

These extensive studies in aqueous methanol glasses are all fully consistent with our preliminary studies that assigned the radical to the N4-protonated species C(N4+H)• (**II**). It is also important to note that the same species can be generated in other non-metal-containing matrices. Preliminary studies on cytidine in aqueous ethylene glycol 1:1 (v/v) also generate the same triplet species, but to reveal this spectrum the matrices have to be annealed to temperatures close to the melting point of the glass. The fact that the same triplet is formed in a different

TABLE 1: Spectral Widths (Δ) of One-Electron-Reduced Cytosine Derivatives in Various Glassy Matrices[a]

compound	matrix	T/K	Δ[b] (mT)	spectrum type
cytosine	CH_3OH/H_2O	130 → n.m.p.[c]	3.5 → 3.2	T[d] → D[e]
	CD_3OD/D_2O	130 → n.m.p.	2.5 (n.c.)[f]	D
	10 M $LiCl/H_2O$	77	3.6	T
	10 M $LiCl/D_2O$	77	2.5	D
cytidine	CH_3OH/H_2O	130 → n.m.p.	3.6 → 2.8	T → D
	CD_3OD/D_2O	130 → n.m.p.	2.5 (n.c.)	D
	10 M $LiCl/H_2O$	77	3.4	T
	10 M $LiCl/D_2O$	77	2.5	D
	$(CH_2OH)_2/H_2O$ (1:1 v/v)	n.m.p.	3.6	T
2′-deoxycytidine (2′-dC)	CH_3OH/H_2O	130 → n.m.p.	3.6 → 2.9	T → D
	CD_3OD/D_2O	130 → n.m.p.	2.5 (n.c.)[d]	D
	10 M $LiCl/H_2O$	77	3.4	T
	10 M $LiCl/D_2O$	77	2.6	D
2′-deoxycytidine 5′-monophosphate (5′-dCMP)	CH_3OH/H_2O	130 → n.m.p.	3.5	T
	CD_3OD/D_2O	130 → n.m.p.	2.5	D
	10 M $LiCl/H_2O$	77	3.6	T
	10 M $LiCl/D_2O$	77	2.6	D
2′-deoxycytidine 3′-monophosphate (3′-dCMP)	CH_3OH/H_2O	130 → n.m.p.	3.6 → 2.8	T → D
		130	2.5	D
N,*N*-dimethyl-2′-deoxycytidine	CH_3OH/H_2O	130 → n.m.p.	2.5 (n.c.)	D
	CD_3OD/D_2O	130 → n.m.p.	2.5	D
	10 M $LiCl/H_2O$	77	2.5	D
	10 M $LiCl/D_2O$	77	2.5	D
cytidine-5,6-d_2	CH_3OH/H_2O	130 → n.m.p.	1.8 → 1.5	D*[g] → S[h]
	CD_3OD/D_2O	130 → n.m.p.	0.75	S
	10 M $LiCl/H_2O$	77	2.15	D
	10 M $LiCl/D_2O$	77	0.9	S

[a] The electron adducts of thymine and uracil have doublet EPR spectra (Δ ≅ 2.3−2.6 mT) irrespective of the type of glass and H_2O or D_2O. [b] Δ = The separation between the low-field up peak and the high-field down peak of the first derivative spectrum. [c] n.m.p. = near melting point. [d] T = triplet spectrum. [e] D = doublet spectrum. [f] n.c. = no change. [g] * = poorly resolved. [h] S = singlet spectrum.

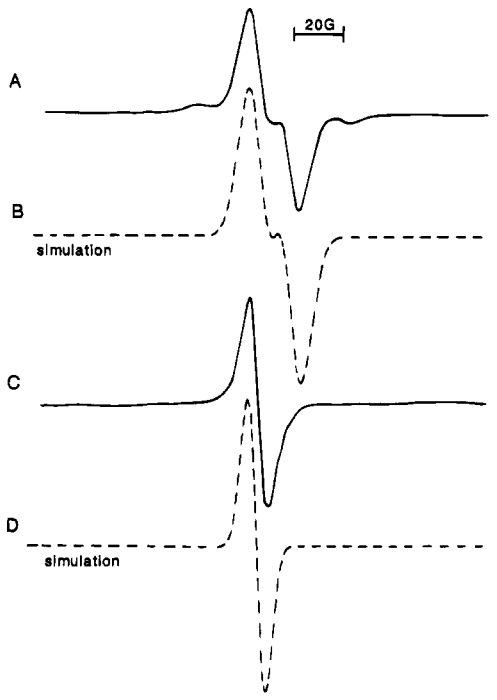

Figure 3. X-Band spectra of cytidine-5,6-d_2 γ-irradiated at 77 K: (A) In $LiCl/H_2O$ showing a poorly resolved doublet spectrum from the added proton at N4 (*ca.* 1.2 mT) and the *ca.* 0.2 mT coupling from C6−D. (B) Simulation of (A) based on parameters in the text. (C) In $LiCl/D_2O$ showing a narrow singlet due to the loss of the proton couplings at C6 and N4. (D) Simulation of (C) based on parameters in the text. All spectra were recorded at 77 K after annealing to 155 K.

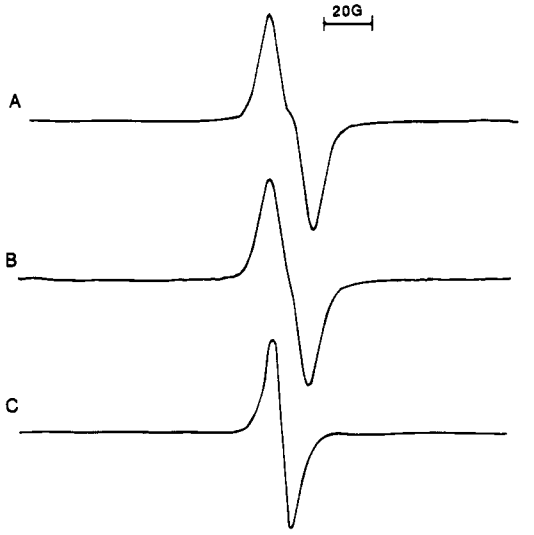

Figure 4. X-Band spectra of cytidine-5,6-d_2 γ-irradiated at 77 K: (A) In $MeOH/H_2O$ after annealing to 130 K showing a poorly resolved doublet similar to that observed in $LiCl/H_2O$. (B) Collapse of the doublet to a singlet on annealing to the melting point of the glass (*cf.* the triplet to doublet conversion in Figure 1A and D). (C) In CD_3OD/D_2O showing a narrow singlet. All spectra were recorded at 77 K and are shown without any modification.

organic matrix strongly supports the assignment of this radical to a cytidine-derived radical rather than a species derived from the matrix.

Microwave Power Saturation. Further evidence that the radical species giving triplet spectra in aqueous LiCl glasses and aqueous methanol glasses are the same was sought from power saturation experiments. Double integration of the triplet EPR spectra for the irradiated samples of 5′-dCMP prepared in each of these matrices recorded at different microwave power levels showed remarkably similar power saturation characteristics. A plot of signal intensity versus (microwave power)$^{1/2}$ for 5′-dCMP in $MeOH/H_2O$, CD_3OD/D_2O, and $LiCl/H_2O$ is displayed in Figure 5. In each of these matrices the signals increase approximately linearly up to microwave power levels of *ca.* 10 μW, but above this, marked power saturation sets in and is accompanied by a concomitant broadening of the spectra

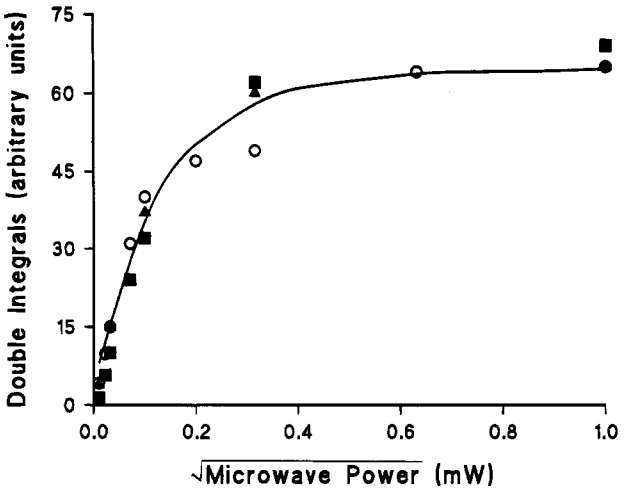

Figure 5. Plot of double integrals of γ-irradiated 5′-dCMP at 77 K as a function of (microwave power)$^{1/2}$, in MeOH/H_2O (■) and CD_3OD/D_2O (○) after annealing to 130 K and LiCl/H_2O (△) after annealing to 155 K. The normalized signal intensities for each matrix (normalized at 5 μW) increase linearly up to a microwave power of *ca.* 10 μW, above which the EPR spectra for the electron adducts become saturated. The similarity of these plots is consistent with the electron adducts of each matrix being the same species.

which eventually become broad unresolved singlets at 10^4 μW. This behavior is very similar to that observed for $C^{\bullet-}$ in duplex systems where the EPR spectrum is a doublet.[11]

Discussion

Our recent observation of triplet EPR spectra arising from one-electron reduction of cytosine and many of its derivatives in protic glasses was quite unexpected, since previous model studies on cytosine had not reported a triplet and formation of $C^{\bullet-}$ on irradiation of protic solutions of DNA, poly[dG-dC], and C-G oligonucleotides is known to give a doublet.[7,14] The reasons for this difference in behavior are far from clear. This present study was initiated to investigate the reasonable proposal that protonation of the amino group occurs because coordination of metal ions from the alkali metal glass with N3 prevents protonation at this preferred site and results in the formation of C(N3$^{\bullet}$••)M^+,N4+H)$^{\bullet}$ (**III**). Clearly if there is unambiguous evidence for N4 protonation of the one-electron-reduced radicals of cytosine and its derivatives in metal-free glasses, then metal ion coordination to N3 is not a necessary condition for N4 protonation and some other explanation must be invoked. Presumably this must also call into question the hypothesis of metal ion coordination blocking N3 protonation even in alkali metal glasses.

The results of irradiation of a range of cytosine derivatives in aqueous methanol glasses are fully consistent with N4 protonation observed in LiCl glasses. This conclusion rests on a number of key observations: (1) The triplet observed for a number of cytosine derivatives irradiated in aqueous methanol glasses is closely similar to that for the species generated in aqueous LiCl glasses (Table 1). (2) The triplet species collapses to a doublet when irradiation is conducted in CD_3OD/D_2O glasses, as seen in aqueous LiCl/D_2O glasses. (3) Irradiation of cytidine-*5,6-d*$_2$ in aqueous methanol leads to a doublet EPR spectrum, as seen in aqueous LiCl glasses, whereas in CD_3OD/D_2O a poorly resolved singlet is observed. (4) Irradiation of *N,N*-dimethyl-2′-deoxycytidine in both aqueous methanol glasses and aqueous LiCl glasses leads to closely similar doublets, suggesting that N4 protonation does not occur for this derivative. (5) Power saturation of the radical species derived from 2′-deoxycytidine giving the triplet spectra in both aqueous methanol and LiCl glasses in our hands occurs at comparable power levels.

Differences between the Q-band EPR spectra for 2′-deoxycytidine irradiated in aqueous methanol glasses and aqueous LiCl at 4 K have been reported by Barnes and Bernhard.[16,17] The former apparently produces a doublet whereas the latter confirms our earlier observations that protonation occurs at N4 even at 4 K. The easiest way to reconcile our observation with those of Barnes and Bernhard is to conclude that in aqueous methanol glasses the radical anion $C^{\bullet-}$ is probably not protonated at 4 K but becomes protonated somewhere in the temperature range 4−130 K. It is difficult to be more precise than this, since it is necessary to anneal samples in aqueous methanol glasses to *ca.* 130 K to remove radicals arising from the matrix and thereby reveal the triplet spectrum. Indeed, Barnes and Bernhard[16] report that a cytosine-dependent triplet signal is obtained following an anneal to 133 K of 2′-dC in aqueous methanol, but they express concern over the high microwave power levels required to saturate this signal. In our hands complete annealing of samples 2′-dC in aqueous methanol is critical in power saturation experiments of the resulting triplet. Power saturation of incompletely annealed samples revealed residual radicals from the matrix that tended to obscure the underlying power saturation of the major triplet. However, carefully annealed aqueous methanol glasses containing 2′-dC showed power saturation characteristics that were very similar to those for the triplet in aqueous LiCl glasses (Figure 5).

The close similarity between the EPR spectra observed for cytosine derivatives in aqueous LiCl glasses and aqueous methanol glasses highlighted above forces the conclusion that in both systems the radical species is C(N4+H)$^{\bullet}$ (**II**), arising from the protonation of the one-electron-reduced cytosine derivative on the exocyclic amino group. This inevitably means that the protonation of N4 in this case cannot be dictated by coordination of metal ions to the preferred protonation site, presumed to be N3. The exocyclic amino group is likely to be well solvated in glassy matrices, and this may mean that there is an appropriately positioned proton that can be readily transferred, even at low temperatures. Thus the N4 protonation should be seen as a kinetic protonation whereas the N3 (or O2) protonation would be the thermodynamic product. Preliminary *ab initio* density functional calculations suggest that the difference in energy between structures **II** and **IV** is approximately 150 kJ mol^{-1} in favor of N3 protonation, i.e. **IV**.[22] It is clear that in oligomeric and polymeric systems the site of protonation is *not* the N4 position even at low temperatures, and it is still not entirely clear why there is this switch. In oligomeric systems where cytosine is involved in a hydrogen-bonded base pair, it is easy to see why a shift of a proton toward N3 from the hydrogen-bonded G competes with N4 protonation seen in monomeric systems. However, our earlier observation that irradiated poly C does *not* protonate predominantely on N4 indicates that base pairing *per se* is not solely responsible for the shift in the site of protonation away from N4.[14] It is probable that solvation of the N4 amino group is different in monomeric and oligomeric systems, and in the absence of any other explanation this may be sufficient to explain the shift in site of protonation.

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References and Notes

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