An EPR Study of the Transfer of Radical-induced Damage from the Base to Sugar in Nucleic Acid Components: Relevance to the Occurrence of Strand-breakage

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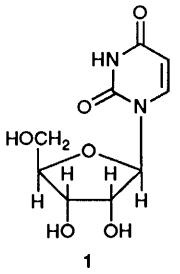
EPR spectroscopy has been employed, in conjunction with a rapid-flow system and the Ti^{III}–H_2O_2 and Ti^{III}–$HOOSO_3^-$ redox couples, to study the reactions of $^{\bullet}OH$ and $SO_4^{\bullet -}$ with pyrimidine nucleosides. From experiments which complement radiolytic and photolytic studies it is concluded that the formation of base-centred radical-cations (from $SO_4^{\bullet -}$ and from $^{\bullet}OH$ at low pH) is followed both by hydration and by loss of the C2′-H in the ribose (but not 2-deoxyribose) ring: further fragmentation follows the latter reaction. The crucial role of the C2′-OH group in the transfer of damage is discussed.

It is generally believed that damage to DNA plays a crucial role in the cytotoxic action of ionizing radiation,[1,2] both *via* direct ionization of the nucleotide bases[3,4] and indirectly from secondary damage *via* reaction of $^{\bullet}OH$ (from water).[5,6] There is increasing evidence that one of the major types of lesion to occur—strand-breakage—results from the formation of radicals in the sugar-phosphate backbone, *via* initial radical-induced damage at the nucleotide bases; reactions of $^{\bullet}OH$ with DNA and its components have been widely investigated (*e.g. via* pulse radiolysis[6–12]) and the results of relevant laser flash photolysis studies (which can bring about direct ionization) have also been reviewed.[13]

Direct evidence for the transfer of damage from pyrimidine moieties to attached ribose sugars in some nucleotides and nucleosides derives in part from EPR studies of the reactions of $^{\bullet}OH$ and $SO_4^{\bullet -}$ (generated photolytically).[14,15] Both of these radicals first attack the alkenic double bond of the pyrimidine base in uridine (**1**) and cytidine and their deoxy counterparts: $SO_4^{\bullet -}$ is believed to generate the appropriate radical-cation which is then responsible for (intramolecular) removal of a hydrogen atom at positions 2 and 3 in the sugar ring in uridine (though not its deoxy counterpart) to give the appropriate hydroxyalkyl radicals. It is also suggested that the subsequent phosphate-loss from the corresponding C2′-centred radical in the appropriate polynucleotide (for which poly-U is a model) provides the appropriate route for strand-breakage.



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We have previously described the use of a continuous-flow method in conjunction with EPR spectroscopy to identify sites of direct attack of $^{\bullet}OH$ on sugar *vs.* base in some nucleotides[16,17] and to study attack and fragmentation reactions in sugars and sugar-phosphates,[18] as well as to establish that acid-catalysed loss of β-OH in radicals of the type $^{\bullet}C$–C(OH) leads to the formation of radical-cations (as with $SO_4^{\bullet -}$ and alkenes).[19] These approaches have also been employed to demonstrate that damage can be transferred from the arene ring in aromatic radical-cations to suitable positions in the side-chains (*e.g.* $^{\bullet +}C_6H_5CH_2CH_2CH_2OH$ gives[20] $C_6H_5CH_2CH_2\dot{C}HOH$ and $^{\bullet +}C_6H_5CH_2CH_2CO_2^-$ gives[21] $C_6H_5CH_2CH_2^{\bullet}$) apparently *via* both through-bond and through-space mechanisms. We now report the results an of an EPR investigation in which we set out to complement the earlier photolytic investigations of the reaction of $^{\bullet}OH$ and $SO_4^{\bullet -}$ with the pyrimidine nucleoside uridine (**1**) and related compounds;[14,15] in particular we wished to ascertain the nature of the sugar-based radicals which are formed and the route by which they are generated (*e.g.* whether through-bond or through-space effects are important). It was also our intention to attempt to rationalize the results of spin-trapping studies which, in comparison with the direct EPR studies referred to above, appear to indicate that more widespread damage to the sugar-ring is involved (see *e.g.* refs. 22–24).

Results and Discussion

Our experiments have been carried out *via* the *in situ* EPR investigations of the reactions of $^{\bullet}OH$ and $SO_4^{\bullet -}$ using a rapid-mixing system in which three streams were mixed shortly (*ca.* 30 ms) before passage through the cavity of the spectrometer: the hydroxyl radical was generated from reaction (1), and $SO_4^{\bullet -}$ *via*

$$Ti^{III} + H_2O_2 \longrightarrow Ti^{IV} + HO^{\bullet} + HO^- \quad (1)$$

reaction (2).[25] In each case the substrate was included in the

$$Ti^{III} + HOOSO_3^- \longrightarrow Ti^{IV} + HO^- + SO_4^{\bullet -} \quad (2)$$

third stream and the pH was adjusted to give the required pH on mixing.

Reactions of $^{\bullet}OH$ *with Pyrimidine Nucleobases and Nucleosides: Continuous-flow Studies.*—Reaction of $^{\bullet}OH$, generated from Ti^{III} and H_2O_2 at pH *ca.* 2, with the nucleobases uracil, thymine, and cytosine gave strong signals from the hydroxyl adducts shown in Table 1 (see also ref. 17 and refs. cited therein); results at pH *ca.* 6 were similar. With uracil and cytosine the C5-adducts **2** and **3** clearly dominate the spectra (though traces of the C6-adduct from uracil can be observed[7,8,17]). For thymine, for which the C5- and C6-adducts **4** and **5** are detected in similar concentrations (as noted on the basis of pulse radiolysis studies[7,8]), the introduction of the C5-methyl group has clearly affected the regioselectivity of attack.

Examination of the reactions of the corresponding nucleo-

Table 1 EPR Parameters of radicals generated from reaction of $^{\bullet}OH$ with pyrimidine nucleobases and nucleosides in aqueous solution[a]

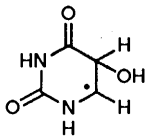
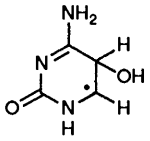
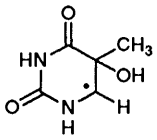
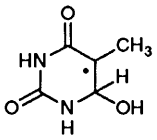
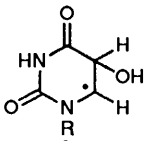
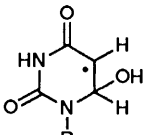
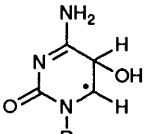
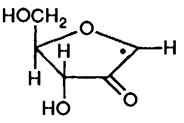
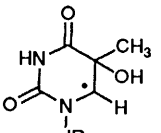
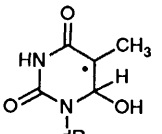
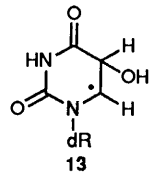
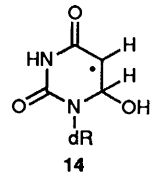
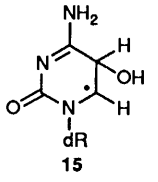
Substrate	Radicals[b]	pH[c]	a(α-H)[d]	a(β-H)[c]	a(other)[d]	g[e]
Uracil	 **2**	2,6	1.842	2.142	0.087 (2 N)	2.0029
Cytosine	 **3**	2,6	1.808	1.803	—	2.0028
Thymine	 **4**	2,6	1.872	—	—	2.0029
	 **5**	2,6	1.533	2.234 (3 H)		2.0032
Uridine	 **6**	2,6	1.862	2.154	0.299 (γ-H)	2.0029
	 **10**	<2	1.995	2.606	0.133 (H) 0.106 (H)	2.0033
Cytidine	 **7**	2,6	1.798	1.798	0.185 (γ-H)	2.0028
	 **11**	<2.5	1.322		0.566 (H) 0.238 (H)	2.0049
Thymidine	 **8**	2,6	1.870			2.0029
	 **9**	2,6		1.085 (1 H) 2.328 (3 H)		2.0032

Table 1 (*continued*)

Substrate	Radicals[b]	pH[c]	$a(\alpha\text{-H})^d$	$a(\beta\text{-}H)^c$	$a(\text{other})^d$	g^e
2-Deoxyuridine	 **13**	2,6	1.862	2.154	0.299 (γ-H)	2.0028
	 **14**	< *ca.* 2	1.037	1.330	—	2.0030
2-Deoxycytidine	 **15**	2,6	1.798	1.798	0.185 (γ-H)	2.0034

[a] For details of the Ti^{III}/H_2O_2 reaction, see text. [b] R = ribose, dR = 2′-deoxyribose. [c] The lower limit in the pH variation was typically *ca.* 1. [d] mT, ±0.01. [e] ±0.0001.

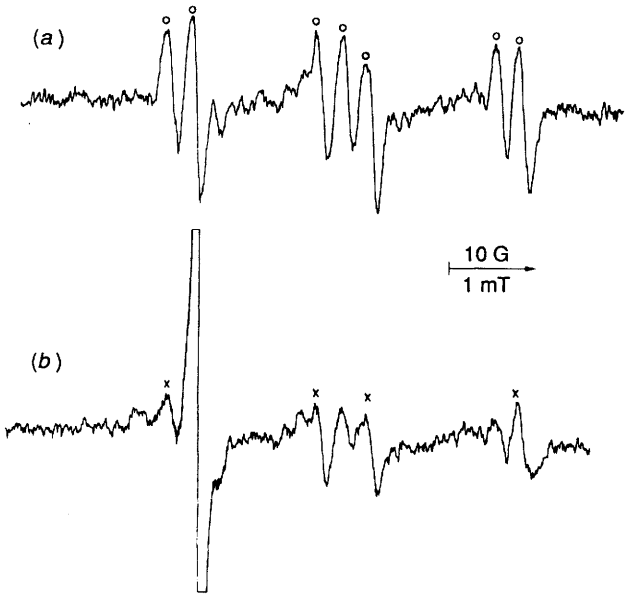
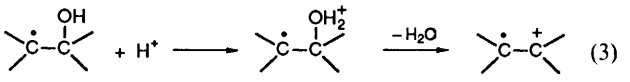


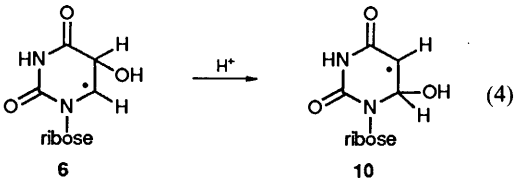
Fig. 1 EPR spectra obtained from 'OH and uridine. (*a*) pH 2: signal attributed to the C5-hydroxyl adduct **6**. (*b*) pH 1: signal (×) attributed to the C6-hydroxyl adduct **10**. The large singlet is from $Ti^{IV}HO_2^{\bullet}$.

sides uridine, thymidine and cytidine under similar conditions led to the detection largely of the corresponding C5-adducts of uridine (**6**), and cytidine (**7**), and to the observation of signals from both C5- and C6-adducts **8** and **9** of thymidine (*cf.* results of a preliminary report of some of this work [17]). The good signal-to-noise ratio [see *e.g.* Fig. 1(*a*)] and our failure to detect traces of signals from sugar-derived radicals, at least for uridine and thymidine, indicates that 'OH attack occurs predominantly, if not exclusively, at the pyrimidine moiety (in agreement with earlier findings[6,17]).

We next repeated these experiments under conditions of increased acidity (pH < 2): as we have previously shown—at least with OH' adducts of alkenes and arenes—protonation of a β-hydroxyl group in a radical can lead to formation of the appropriate radical-cation *via* loss of water [reaction (3)].[19]


(3)

Several clear changes could be monitored for the nucleosides and the nucleobases themselves. Thus for uridine the signal from the C5-adduct became accompanied (at pH < 2) by a signal, characterized by a doublet (1.995 mT) of doublets (2.606 mT) and further hyperfine splittings [and with *g* 2.0033: see Fig. 1(*b*)]: by pH 1 the ratio of the two signals was *ca.* 1:1. This radical has previously been detected following reactions of $SO_4^{\bullet -}$ with uridine[14,15] (see also below) and assigned to the C3′ sugar-derived radical. However, on the basis of the hyperfine splittings, and for mechanistic and other reasons described below, we suggest an alternative assignment of this species to the C6-adduct **10**, formed *via* acid-catalysed transformation of the C5-isomer [reaction (4)]. With uracil itself the signal from **2** became weaker, but no new signals could be unambiguously detected.


(4)

With thymine and thymidine no new radicals appeared on lowering the pH to *ca.* 1 though the overall intensities decreased somewhat. However, as the pH was lowered (< 2.5) in corresponding reactions of cytidine a weak spectrum, assigned to the sugar-derived radical **11** became clearly detected (see Fig. 2); we have previously detected and characterized[16,17] this species in the reaction of adenosine and adenosine-5-phosphate with 'OH and it has been reported in reactions of $SO_4^{\bullet -}$ with uridine and cytidine[14,15] (see also below). As argued previously, this radical, which was not detectable at higher pH, is believed to be derived *via* an acid-catalysed reaction involving formation of the corresponding C2′-sugar radical **12** from the protonated

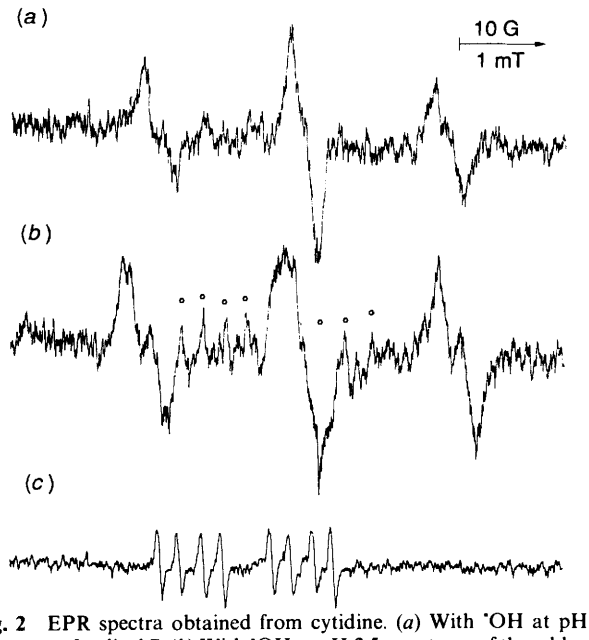


Fig. 2 EPR spectra obtained from cytidine. (*a*) With $^{\bullet}OH$ at pH 6: spectrum of radical **7**. (*b*) With $^{\bullet}OH$ at pH 2.5: spectrum of the adduct **7** accompanied by that from the sugar-derived radical **11**. (*c*) Spectrum of **11** obtained from the reaction of $SO_4^{\bullet-}$ at pH 2.5.

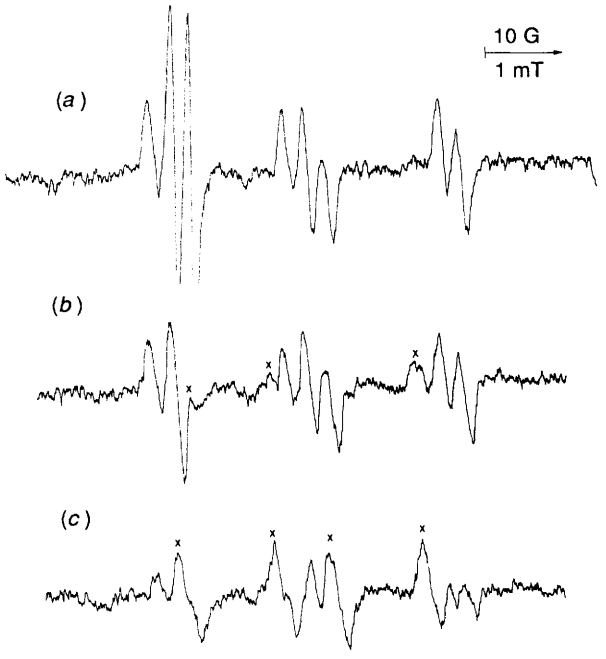
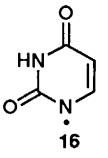
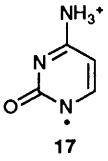
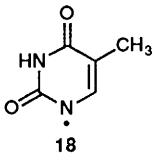
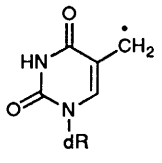
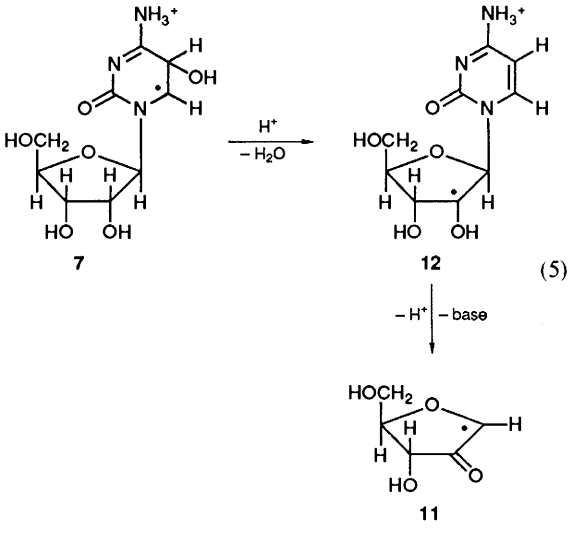


Fig. 3 EPR spectra obtained from 2′-deoxyuridine. (*a*) With $^{\bullet}OH$ at pH 2: signal from the C5-hydroxyl adduct **13**. (*b*) With $^{\bullet}OH$ at pH 1.5: signal from the C5-hydroxyl adduct **13** and the C6-hydroxyl adduct **14**. (*c*) With $SO_4^{\bullet-}$ at pH 1: signal from the C6-hydroxyl adduct **14** (×).

Table 2 Radicals detected by EPR spectroscopy in continuous-flow experiments with $SO_4^{\bullet-}$ in aqueous solution[a,b]

Substrate	Radical	*a*(N)	*a*(H)	*g*
Uracil	 **16**	0.503	1.058 0.132	2.0043
Cytosine	 **17**	0.463	1.799	2.0040
Thymine	 **18**	0.489	1.905 (3 H)	2.0053
Uridine	**10, 11**			
Cytidine	**11**			
Thymidine	 **19**		1.601 1.030 1.510	2.0023
2-Deoxyuridine	**13, 14**			
2-Deoxycytidine	**15**			

[a] For details of $SO_4^{\bullet-}$ generation, see text. [b] pH *ca.* 2.



(5)

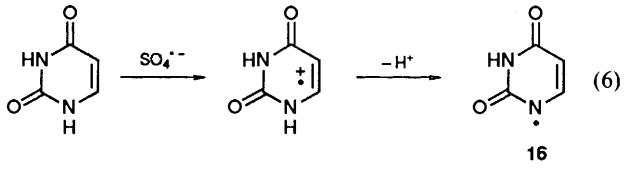
form of cytidine (pK_a 4.3) and subsequent loss of both the α-hydroxy-group proton (on the sugar) and the base [*cf.* the rearrangement of other α,β-disubstitued radicals with an oxygen (+M) substituent at C_α and a good leaving group in the β-position[14–18]]. For cytosine itself the adduct-radical **3** was the only species detected (in decreasing intensity) as the pH was lowered.

Similar experiments were carried out with the nucleosides 2′-deoxyuridine and 2′-deoxycytidine. For the former (Fig. 3), the spectrum of the C5-adduct **13**, detected as the sole species present with $^{\bullet}OH$ at pH 2, became accompanied by a further doublet of doublets [*a*(H) 1.862, 2.154 mT; *g* = 2.0033] as the pH was lowered. On the basis of the observed splittings and the parallel behaviour of uridine itself we assign the latter to the corresponding C6-OH adduct **14** (presumably formed *via* acid-catalysed loss of the hydroxy group and rehydration; see later); it is clearly not a C3′-derived radical, which would have *three* β-proton splittings. For 2′-deoxycytidine and $^{\bullet}OH$, only the C5-adduct **15** was observed; there was no significant change with pH, and no sign of a sugar-derived radical (in contrast to the behaviour of cytidine itself).

Reactions of $SO_4^{\bullet-}$ *with Pyrimidine Nucleobases and Nucleosides: Continuous-flow Studies.*—In order to support the

assignments and mechanistic deductions made above we next studied the reactions of the bases and their derivatives with $SO_4^{\bullet -}$, which is believed to generate the appropriate radical-cation *via* reaction with the alkene double bond in the base.[14,15] Experiments were typically carried out at pH *ca.* 2–4, but some experiments were repeated at lower pH.

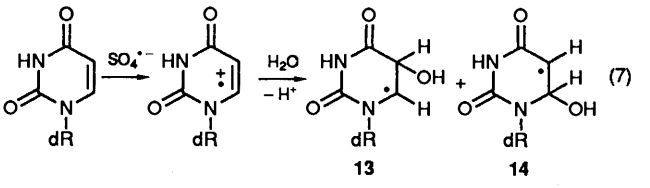
The observations of the EPR signal from the radical **16** from uracil under these conditions is consistent with these proposals and with previous pulse radiolysis studies:[12] it evidently results from the rapid deprotonation of the first-formed radical-cation [reaction (6)]. Failure to detect **16** in reactions of $^{\bullet}OH$ at lower pH is believed to reflect the slower rate of generation of the radical-cation *via* protonation of the $^{\bullet}OH$ adduct in the latter system.



(6)

16

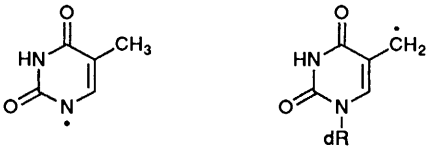
Under similar conditions uridine gave a signal which clearly characterizes the formation of the sugar-derived radical **11** described above, together with the strong signal identical to that obtained with $^{\bullet}OH$ at low pH and assigned by us to the C6-OH adduct **10**. 2′-Deoxyuridine gave spectra from two radicals assigned to the C5- and C6-hydroxyl adducts **13** and **14** [reaction (7)], exactly as noted for $^{\bullet}OH$ attack at low pH. Several points based upon these particular observations are worthy of special note: firstly, the similarity of the behaviour induced by the $^{\bullet}OH/H^+$ and $SO_4^{\bullet -}$ reactions (which would be expected if radical-cations are formed in both processes); secondly, the clear indication that *hydration* of the radical-cation (from $SO_4^{\bullet -}$) is involved [see *e.g.* reaction (7) and ref. 10]; thirdly the clear evidence that the new spectrum obtained at low pH (and with $SO_4^{\bullet -}$) is **14** and not a sugar-derived radical (had this case been the case the presence of –ĊH or $-CH_2$ at C2 would have been revealed by the splittings). We believe this last observation provides crucial evidence for the correct identification of a base-derived radical **10** in the corresponding reaction of uridine.



(7)

13 **14**

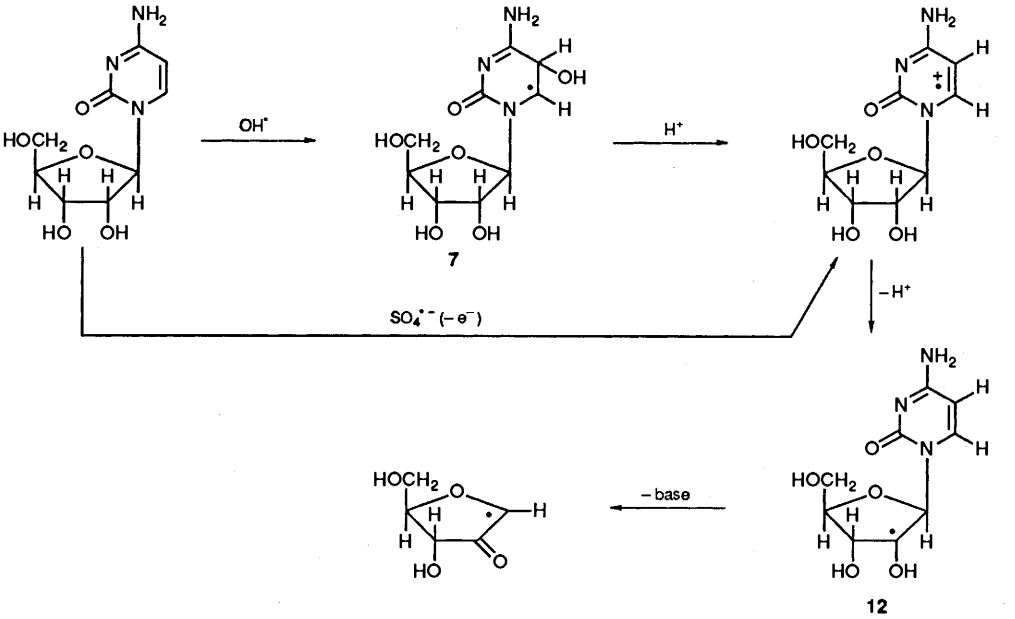
Cytosine reacted with $SO_4^{\bullet -}$ to give weak signals attributed to **17** (see Table 2), evidently formed by deprotonation of the first-formed radical-cation (see also ref. 8). Cytidine gave only the sugar-derived radical **11**, whereas 2′-deoxycytidine gave the appropriate C5-OH adduct **15**. For thymine itself the reaction with $SO_4^{\bullet -}$ gave an EPR signal from the radical **18** (see Table 2), the 2′-deoxynucleoside thymidine reacted with $SO_4^{\bullet -}$ to give largely the C6-hydroxyl adduct **8**, as observed for $^{\bullet}OH$ attack, together with signals from the allylic radical **19**, formed *via* deprotonation at carbon [*cf.* reaction (6) and refs. 9, 11].



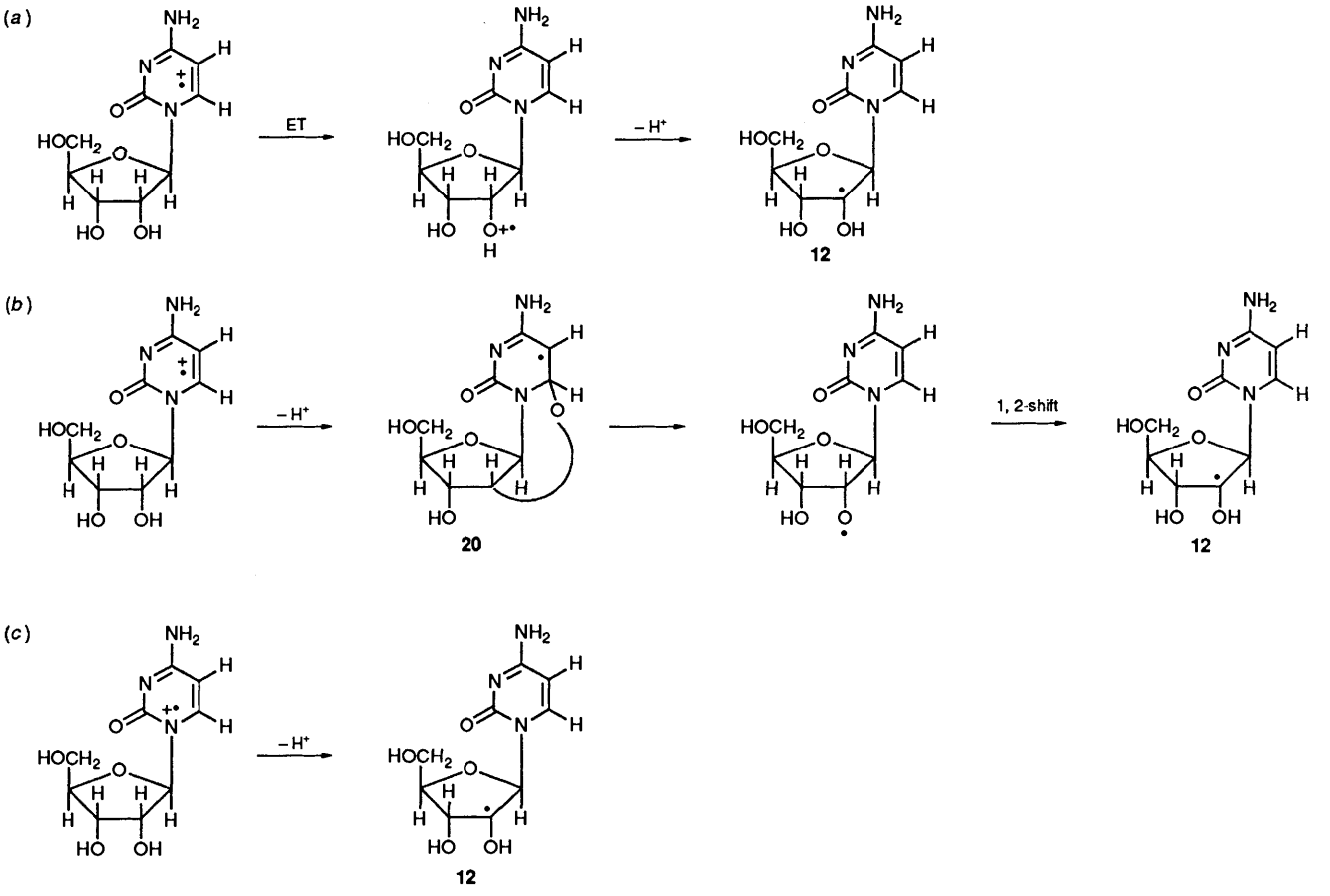
18 **19**

Mechanistic Interpretation.—Our observations for the reaction of $SO_4^{\bullet -}$, together with the results for $^{\bullet}OH$, appear to provide a firm basis for a (largely) unified mechanism in which radical-cations (from $SO_4^{\bullet -}$ or protonated hydroxyl adducts) react *via* a variety of pathways. These include deprotonation at nitrogen (for uracil), deprotonation of the methyl substituent on the pyrimidine ring (thymidine), hydration of the carbon–carbon double bond in the pyrimidine ring (especially prominent for the 2′-deoxynucleosides) and, in some cases, transfer of damage to the sugar ring. Instances of the latter appear to be limited to the *ribose* derivatives (cytidine, uridine), rather than their deoxyribose counterparts, and then to give only the C2′-derived radical **11**; this reaction (see Scheme 1) is more prominent with cytidine than uridine, for the latter of which it is suggested that hydration is also important.

If our structural and mechanistic interpretations are correct



Scheme 1



Scheme 2

then it follows that the transfer of damage to the sugar ring in pyrimidine nucleosides occurs only to the C2′-position in a reaction which is associated with the OH substituent at that position (*n.b.* our interpretation differs from that of Hildenbrand and coworkers [14,15] whose alternative assignment of the radicals from uridine requires the additional occurrence of damage at C3′). The transfer of damage to C2′ (which leads to base loss, and, presumably, phosphate loss in the C3′-phosphate derivatives) may be attributable to one or more factors including the ability of the C2′-OH substituent to stabilize the intermediate radical **12** (as well as to allow subsequent irreversible elimination of base) and the possible involvement of the C2′-oxygen atom in damage transfer.

Three more detailed mechanistic possibilities are outlined in Scheme 2 (illustrated for cytidine). In the first [Scheme 2(*a*)] electron-transfer to the pyrimidine-centred radical-cation from the C2′-oxygen is followed by rapid deprotonation at C2′. In the second [Scheme 2(b)] nucleophilic attack by the C2′-hydroxy group on the 'alkene' radical-cation (to give **20**) is followed by β-fission, to give an alkoxyl radical which may undergo a rapid 1,2-hydrogen shift. In the third, more direct oxidation of C2′-H occurs, for example *via* reaction of an amine-centred radical-cation, which might be responsible for hydrogen-atom transfer [as shown in Scheme 2(*c*)].

The following related observations may be relevant. The EPR analysis of anisotropic spectra from matrix-isolated radical-cations from *N*,*N*-dimethyluracil and *N*,*N*-dimethylthymine indicate [26] that the spin- and charge-density is largely localized in the C5–C6 (alkene-like) portion of the molecule [*cf.* Scheme 2(*a*) rather than 2(*c*)]. Clear analogies exist for the loss of HO-activated C–H bonds in radical-cations of aromatic side-chain alcohols *via* nucleophilic ring-closure and subsequent ring-opening (*e.g.* $^{\bullet+}PhCH_2CH_2CH_2OH$ to $PhCH_2CH_2\dot{C}HOH$) *via* a mechanism in which the well-established 1,2-shift occurs [as outlined in Scheme 2(*b*)].[20] Other support for the mechanism derives from the observation that alkylation of the C2′-OH group (in 2′,3′-*O*-isopropylidene-uridine and -cytidine [14]) appears to prevent the loss of C2′-H.

Although our conclusions must remain somewhat tentative at this stage we propose that the most likely mechanism for damage transfer involves approach of C2′-OH to the double bond (models indicate a separation of *ca.* 0.3 nm of the C2-oxygen and C6) followed either by direct electron-transfer or by nucleophilic attack and ring-opening. The appropriate intermediate **20** in the latter route might well then be that responsible for the transient absorptions (with $t_{\frac{1}{2}}$ 11 μs) at λ 345 and 530 nm observed [15] in the laser flash photolysis of cytidine (a similar transient was detected for uridine but not the deoxy- or isopropylidene-analogues). Lastly we note that related mechanisms would also account for the transfer of damage in adenosine and its derivatives: our observation [16,17] in the reaction of adenosine with $^{\bullet}OH$ of the C4′- and C5′-derived radicals, as well as C2′-derived species, implies that overall electron-transfer from the (larger) base to the ribose ring-oxygen and C5-OH is followed by deprotonation at C4′ and C5′ respectively.

The implications for longer-range transfer of damage to sugar molecules in *neighbouring* nucleotides will be explored by applying these approaches (together with spin-trapping techniques) to related dinucleotides and the polynucleotides and nucleic acids themselves.

Experimental

Experiments were carried out with a Bruker ESP 300 EPR spectrometer and a three-way continuous flow system, as described previously.[25] For the generation of $^{\bullet}OH$ at pH *ca.* 2,

stream (i) contained Ti^{III} ($TiCl_3$; 5×10^{-3} mol dm^{-3}) and H_2SO_4 (to achieve the appropriate final pH), stream (ii) contained H_2O_2 (typically 5×10^{-2} mol dm^{-3}), and the substrate (typically 10^{-2} mol dm^{-3}) and NH_3 were contained in stream (iii). For experiments at pH 6, EDTA (5×10^{-3} mol dm^{-3}) was added to to the Ti^{III} in stream (i). Experiments with $SO_4^{\bullet -}$ employed $Ti_2(SO_4)_3$ (5×10^{-3} mol dm^{-3}) and H_2SO_4 in stream (i) and $HOOSO_3^-$ (typically 8×10^{-2} mol dm^{-3}, added as 'oxone', potassium peroxymonosulphate, $2KHSO_3$, $KHSO_4$, K_2SO_4) in stream (ii). All solutions were thoroughly deoxygenated (with a stream of nitrogen).

Chemicals used were commercial samples (Sigma), used as supplied.

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