Radical Combination Processes under the Direct Effects of Gamma Radiation on Thymidine

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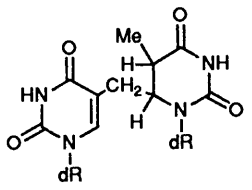
The isolation and structural analysis of a variety of dimeric thymidine products produced by the direct effects of γ-radiation on thymidine in frozen aqueous solution are described. These products may, with the exception of the spore photoproducts, be accounted for by the simple combination of various primary and secondary radicals. As well as being thymidine lesions possibly formed in cellular DNA and therefore of biological interest, from a mechanistic point of view, the formation of these adducts provides good support for the formation of the respective free-radical precursors. In particular, evidence is provided for the formation of the N(3)-centred radical formed by deprotonation of the thymidine radical cation at this position. Other important radicals formed are centred at the C(5), C(6), C(5'), and the methyl-group positions.

The harmful effects of ionising radiation (cell lethality, mutagenesis, and carcinogenesis) may in part be associated with chemical modification of cellular DNA in the form of strand breaks,[1] base and sugar modifications,[2] and DNA-protein crosslinks.[3] The mechanisms by which these alterations are produced may broadly be divided into two groups resulting from the direct and indirect effects of the incident radiation. The indirect effects concern the radiolysis of the bulk solvent, usually water, to give reactive species such as hydrogen atoms, hydroxyl radicals, and aqueous electrons which subsequently attack the substrate. Since the cell has been considered an essentially aqueous system, the indirect effects have received by far the greater proportion of the research into this field, such that now a variety of possible lesions are known (from model studies—for recent reviews see refs. 4–8) and some of these lesions have been detected in isolated[9–12] and cellular DNA.[13–16] It has, however, been shown that the environment of nuclear DNA is far from that of an ideal aqueous solution and that there is little free water in its immediate proximity. As a result, the direct effects[17–19] may make a significant contribution to the overall damage induced in DNA by ionising radiations *in vivo*.[20]

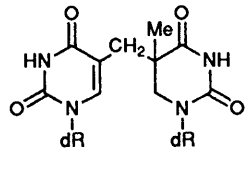
The nature of the primary and secondary radical species formed upon exposure of DNA and its model compounds to ionising radiations has been studied in detail using ESR and electron nuclear double resonance (ENDOR) techniques (for a review see ref. 21); however, the identities of the final diamagnetic progeny of these intermediate radicals remain for the most part unknown. We have recently reported results of a study of the direct effects of ^{60}Co γ-radiation on thymidine using the frozen aqueous solution system.[22,23] Under these conditions, the diffusion of water radicals to the solute is minimised, resulting in predominantly direct effects processes. Since then we have completed the structural analysis of a series of adducts also isolated during that study, the details of which will be reported here. It is noteworthy that the dimeric pyrimidine decomposition products that we have isolated are different to those found to be formed under the indirect effects of ionising radiation,[24–28] which result from the attack of water radiolysis products on the substrate.

Results and Discussion

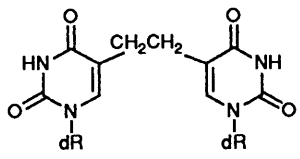
The products described below were isolated and purified using reversed-phase and silica gel HPLC. They may be separated into six classes based on structural features. The structural analysis of these products relied heavily on Fast Atom Bombardment (FAB) mass spectrometry, ^{1}H NMR spectrometry and, in some cases, ^{13}C NMR spectrometry. The compounds were all analysed by 2D homonuclear chemical-shift correlation (COSY) NMR to help in the assignment of the one-dimensional spectrum signals, and all were subsequently iterated using the LAOCOON III program, again verifying signal attributions and refining chemical-shift and coupling-constant data. The ^{1}H NMR data for the compounds are listed in Table 1. The nomenclature used in the trivial nomenclature of these products is that used in ref. 29.



1a, b



2a, b



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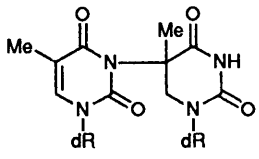


4a, b

Quantitatively, the sum of the yields of the dimeric compounds reported here corresponds to *ca.* 5% of the overall 10% thymidine degradation at the doses used. The remaining proportion of the degradation is accounted for by monomeric thymidine derivatives.[22,23]

(5R*,6R*)- *and* (5S*,6S*)-6-(α-*Thymidyl*)-5,6-*dihydrothymidine* **1a** *and* **1b**.—This is quantitatively the most important of the thymidine adducts formed under the direct effects of γ-radiation on thymidine. They are major lesions of the direct effects being formed in a yield as much as a third of that of the quantitatively most important lesion products, (5*R*)- and (5*S*)-

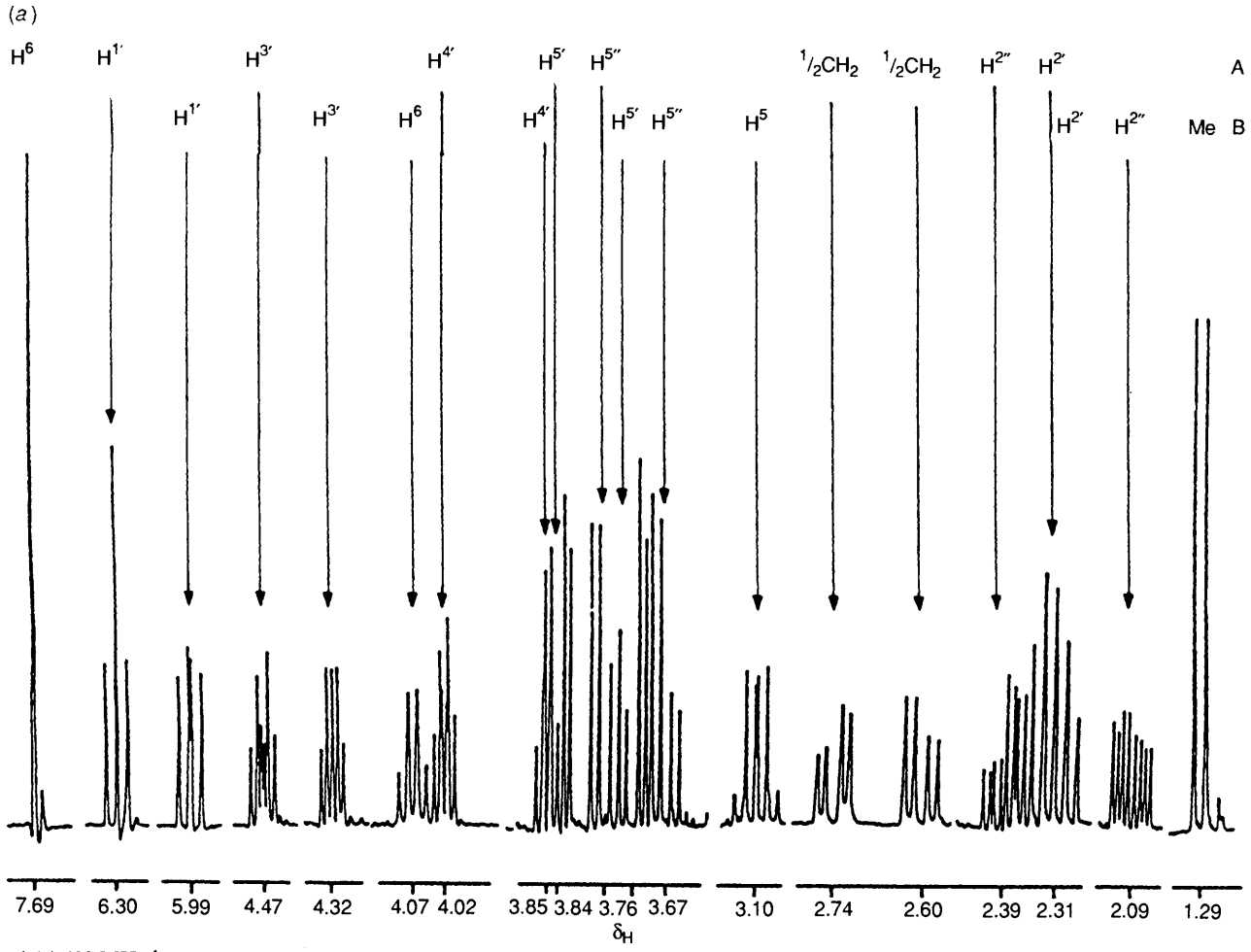


Fig. 1 (*a*) 400 MHz ^{1}H NMR spectrum for compound **1a** recorded in D_2O *vs.* TSP internal standard. For A and B see Table 1

5,6-dihydrothymidines,[22] and represents *ca.* 30% of the total thymidine decomposition. The molecule has asymmetric centres at C(5) and C(6) of the saturated base residue; however, only two of the possible four diastereoisomers are formed, for which we shall give a likely explanation later.

Both isomers were shown by FAB mass spectrometry to have molecular masses of 484 amu, twice that of the parent thymidine. The UV absorption spectrum indicated a λ_{max} of 262 nm, suggesting unsaturation of at least one of the base residues. The two diastereoisomers are distinguished by ^{1}H NMR spectrometry, where both molecules show the same set of proton signals but with different chemical shifts and coupling patterns. The isomer eluting first from a reversed-phase HPLC column **1a** is formed in a yield approximately twice that of the isomer eluting second. The ^{1}H NMR spectra [Figs. 1(*a*) and 1(*b*)] indicate for both isomers the presence of two intact osidic residues. The indication of the α-6 linkage comes from a study of the base proton signals. We note the presence of only one vinyl proton signal in each case, indicating one of the base residues to be saturated. The presence of only one methyl signal indicates that the bridge is *via* a methyl group. The fact that this signal is upfield of the chemical shift normally expected for methyl groups attached to a vinylic carbon and that it is seen as a doublet suggests that it is attached to the saturated residue. The 2D COSY experiment showed that the methyl group is coupled to a proton having a chemical shift similar to the C(5) proton of 5,6-dihydrothymidine.[30] The other methyl signal has collapsed and has been replaced by the AB part of an ABX system at δ 2.7, X being the (6)H signal of the saturated pyrimidine moiety whose signal can be seen at δ 4.1. This strongly suggests that the α-thymidyl group is linked to C(6) of the saturated pyrimidine moiety.

A nuclear Overhauser effect (NOE) difference spectrum was recorded for each isomer. Upon saturation of the vinyl proton resonance, an NOE enhancement was detected on the (1′)H, (2′)H, and (3′)H signals of the downfield group of osidic protons, and we were hence able to link a particular set of osidic signals to a particular base residue. Saturation of the methyl resonance yielded an NOE response on the methyne (6)H proton signal in both cases. This suggests that the methyl group and the (6)H proton are *cis* to each other and therefore that the two diastereoisomers are 5*R**,6*R** and 5*S**,6*S**. As yet it has not been possible to assign the absolute configuration to a particular isomer.

Mechanistically, three possible alternatives exist for the formation of compounds **1a** and **1b**, either the straightforward combination of a 5-(2′-deoxymidinyl)methyl (dThd$^\alpha$) radical with a 5,6-dihydrothymidin-6-yl (hdThd-6) radical, attack of a dThd$^\alpha$ radical at C(6) of an adjacent thymidine molecule with addition of hydrogen ($e^- + H^+$) to C(5), or possibly a concerted process. In order to differentiate between consecutive and concerted mechanisms, we carried out two experiments using deuterium labelling. The first involved the irradiation of thymidine in frozen D_2O solution. In our earlier paper,[22] we showed in a similar experiment that the proton added to C(5) during the formation of 5,6-dihydrothymidine was derived from hydration water and that deuterium was incorporated at this position when thymidine was irradiated in deuterium oxide.

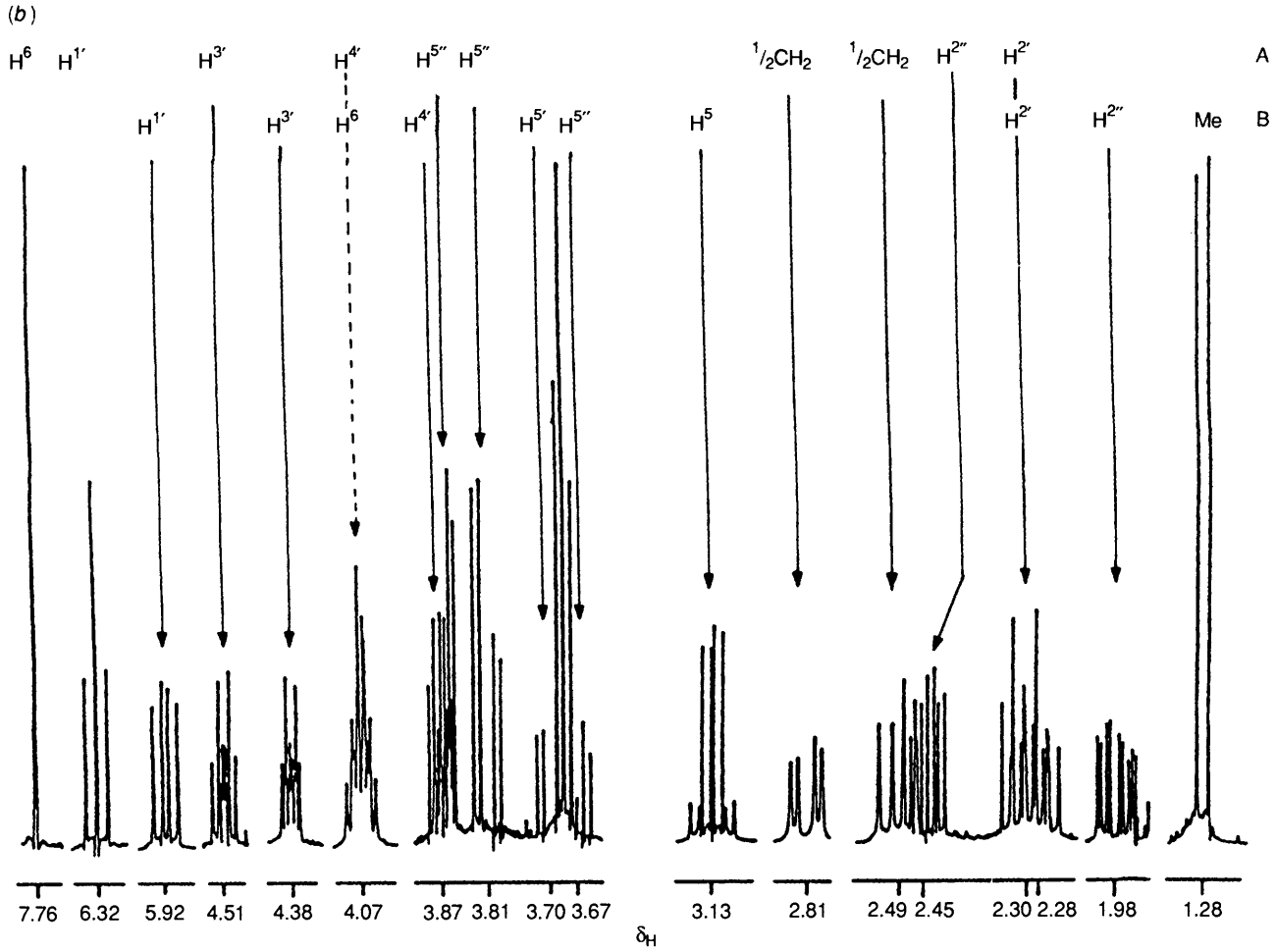


Fig. 1 (*b*) 400 MHz ^{1}H NMR spectrum for compound **1b** recorded in D_2O *vs.* TSP internal standard

We would expect a possible 5-yl radical formed in the attack of a dThd$^\alpha$ radical at C(6) of a thymidine molecule to behave in a similar way and therefore that the ^{1}H NMR spectrum of the resulting product would show incorporation of deuterium. This was shown to be the case for *ca.* 50% for each isomer. If the mechanism proceeds *via* the 5-yl radical, we would expect 100% deuterium incorporation at C(5), as was shown to be the case for the 5,6-dihydrothymidines. The most likely explanation for the observation of only 50% deuterium incorporation is that when initially formed, all four possible isomers are present with a hydrogen, not deuterium, atom at C(5). During annealing, keto–enol tautomerism allows the methyl group of molecules, where this group is *cis* to the thymidyl residue at C(6), to epimerise to yield one of the two apparently favoured *trans* configurations. During this epimerisation, deuterium would be incorporated at C(5). Those molecules originally formed in one of the favoured conformations prior to annealing would retain hydrogen at C(5). We feel that we can rule out the possibility of addition of a dThd$^\alpha$ radical at C(6) of thymidine.

Our second probe of the mechanism involved concerned the irradiation of thymidine deuteriated at the methyl group. In this way we could determine whether or not the mechanism was a concerted one with transfer of a methyl proton from one thymidine molecule to C(5) of a neighbouring one. The ^{1}H NMR spectra of the products obtained during this study indicated the presence of a C(5) proton and we also observed a clear coupling with the (6)H proton. The remaining possibility is radical combination. The formation of the dThd$^\alpha$ radical is undoubtedly due to deprotonation of the initially generated thymidine radical cation; however, this is the first time that we have found evidence in this study for the formation of an hdThd-6 radical. Protonation of the thymidine radical anion occurs exclusively at C(6) to yield the 5,6-dihydrothymidin-5-yl (hdThd-5) radical. The hdThd-6 radical must be formed by hydrogen-atom transfer, and in addition the transfer must occur from a non-exchangeable proton site within a neighbouring molecule, possibly from C(5'), the 5,6-dihydro-5',6-cyclothymidines being quantitatively important products under these conditions.[23] Hydrogen atoms have been shown to add preferentially to C(5) of thymidine photolysed in aqueous solution.[31] Although not demonstrated unambiguously, these experiments suggest that the likely mechanism for the formation of these products involves a radical-combination process.

(5R*)- *and* (5S*)-5-(α-*Thymidyl*)-5,6-*dihydrothymidine* **2a** *and* **2b**.—Both diastereoisomers of the spore photoproduct have been isolated during this study. The FAB mass spectra confirmed the molecular masses at 484 amu, and the UV spectra showed λ_{max} 270 nm for each, indicating that at least one of the base residues was still unsaturated. The two diastereoisomers gave similar ^{1}H NMR features (Table 1), each indicating the presence of one vinylic proton and one methyl group at a chemical shift inconsistent with it being attached to an ethylenic carbon. The proposal of a linkage involving the C(5) carbon is supported by the observation of no vicinal coupling to the

Table 1 400 MHz ^{1}H NMR chemical shifts (δ_H) and coupling constants (J/Hz) for the new thymidine adducts. Chemical shifts are given relative to internal TSP and are accurate to 0.01 ppm. Coupling constants are accurate to within 0.1 Hz

	1a		**1b**		**2a**		**2b**		
	A	B	A	B	A	B	A	B	**3**
δ(1′)H	6.30	5.99	6.32	5.92	6.28(4)	6.28(0)	6.29	6.25	6.29
δ(2′)H	2.31	2.31	2.30	2.28	2.34	2.31	2.34	2.28	2.28
δ(2″)H	2.39	2.09	2.45	1.98	2.44	2.15	2.42	2.14	2.41
δ(3′)H	4.47	4.32	4.51	4.38	4.49	4.38	4.48	4.38	4.46
δ(4′)H	4.02	3.85	4.07	3.87	4.07	3.90	4.04	3.91	4.05
δ(5′)H	3.84	3.72	3.87	3.70	3.87	3.74	3.85	3.77	3.83
δ(5″)H	3.76	3.67	3.81	3.67	3.80	3.67	3.77	3.70	3.76
δMe		1.29		1.28		1.28		1.16	
δCH_2	2.74		2.81		2.90		2.85		2.57
δCH_2	2.60		2.49		2.52		2.52		2.57
δCH_2						3.38		3.43	
δCH_2						3.38		3.39	
δCH_2	3.10		3.13						
δ(6)H(s)[a]		4.07		4.07					
δ(6)H(u)[a]	7.69		7.76		7.83		7.70		7.62
$J_{1',2'}$	6.7	7.9	6.9	9.4	6.6	7.8	6.7	7.9	6.7
$J_{1',2''}$	6.7	6.2	6.5	5.7	6.5	6.6	6.6	6.5	6.7
$J_{2',2''}$	−14.6	−13.9	−14.2	−13.6	−14.1	−14.1	−14.1	−14.1	−14.1
$J_{2',3'}$	6.6	6.4	6.4	6.2	6.3	6.7	6.5	6.5	6.4
$J_{2'',3'}$	4.3	3.4	4.1	2.3	4.2	3.7	4.3	3.6	4.0
$J_{3',4'}$	3.9	3.8	3.9	2.4	3.8	3.5	4.0	3.5	3.8
$J_{4',5'}$	3.6	4.3	3.2	3.9	3.4	4.0	3.5	4.2	3.6
$J_{4',5''}$	5.3	5.5	4.3	4.5	4.5	5.3	4.6	5.4	4.5
$J_{5',5''}$	−12.5	−12.1	−12.6	−12.2	−12.5	−12.3	−12.5	−12.2	−12.4
$J_{5,6}$	5.7	5.4							
$J_{Me,5}$	7.1	7.1							
$J_{CH_2,6}$	6.2	8.2							
$J_{CH_2,6}$	4.5	4.2							
J_{CH_2gem}	−14.5		−14.4		−14.3		−14.4		
J_{CH_2gem}							−13.1		

	4a		**4b**		**5a**		**5b**		**6**	
	A	B	A	B	A	B	A	B	A	B
δ(1′)H	6.29	6.23	6.28	6.23	6.32	6.27	6.31	5.66	6.27	6.27
δ(2′)H	2.32	2.33	2.18	2.30	2.40	2.34	2.50	2.79	2.41	2.34
δ(2″)H	2.18	2.50	2.10	2.50	2.32	2.21	2.32	2.19	2.40	2.43
δ(3′)H	4.34	4.47	4.32	4.43	4.48	4.36	4.57	4.39	4.50	4.46
δ(4′)H	3.85	4.09	3.92	4.09	4.04	3.95	3.93	3.87	4.12	4.06
δ(5′)H	3.68	3.88	3.76	3.87	4.29	3.78	3.82	3.80	3.86	3.84
δ(5″)H	3.56	3.78	3.70	3.78		3.70		3.70	3.72	3.76
δMe		2.02		2.03	1.90		1.92		1.84	
δMe	1.79		1.76			1.29		1.24		
δCH_2	4.39		4.38						4.38	
δCH_2	3.46		3.51						4.35	
δ(5)H						3.11		3.22		
δ(6)H(s)[a]						3.82		3.98	7.58	
δ(6)H(u)[a]	8.02		8.03		7.92		7.45		7.97	
$J_{1',2'}$	7.7	6.4	7.9	6.5	6.7	8.1	8.7	7.3	6.6	6.7
$J_{1',2''}$	7.6	6.4	6.5	6.5	6.7	6.3	5.9	7.0	6.6	6.5
$J_{2',2''}$	−14.3	−14.1	−14.1	−14.1	−14.0	−13.9	−14.3	−13.5	−14.0	−14.1
$J_{2',3'}$	6.6	6.6	6.4	6.6	6.8	6.9	6.2	7.3	8.0	6.7
$J_{2'',3'}$	3.9	4.6	3.6	4.5	4.4	3.4	2.1	4.0	3.1	4.1
$J_{3',4'}$	3.8	4.6	3.7	4.5	4.1	3.5	2.0	4.5	4.2	3.9
$J_{4',5'}$	4.0	3.5	4.0	3.6	2.3	4.0	9.7	3.9	3.0	3.5
$J_{4',5''}$	5.5	5.1	5.5	5.3		5.6		6.3	5.0	4.9
$J_{5',5''}$	−12.3	−12.6	−12.2	−12.5		−12.1		−12.0	−11.3	−12.4
$J_{5',6}$					2.1		1.9			
$J_{5,6}$					1.2		6.4			
$J_{Me,5}$					7.4		7.1			
$J_{Me,6}$	1.0		1.1		1.1		1.1		1.2	
J_{CH_2gem}	−12.6		−11.8						−12.1	

[a] u and s refer to the unsaturated and saturated thymine residues, respectively. Generally, where applicable, A denotes the unsaturated thymidine residue signals, and B denotes the saturated thymidine residue signals.

methyl protons, and by the presence of a two-proton signal seen as either a singlet or a tight AB quartet in the region of δ 3.4 assigned as the ring CH_2 group at the 6-position of the saturated base. The methylene bridge was also seen as an AB quartet at a chemical shift similar to that observed for the AB part of the corresponding ABX system of 6-(α-thymidyl)-5,6-dihydrothymidine [dThd(α-6)hdThd].

The mechanism for the formation of these products may also proceed *via* a consecutive or a concerted process. The irradiation of thymidine deuteriated at the methyl group indicated that the methyl proton was transferred apparently quantitatively to C(6) of the eventual saturated base residue. The mechanism is hence a concerted one and does not appear to proceed *via* the combination of a $dThd^{\alpha}$ radical with an hdThd-5 radical. In our earlier work,[22] we showed that the cyclobutane-type dimers are also products of the direct effects of γ-radiation on thymidine, and the formation of the spore photoproducts is undoubtedly the result of excitation processes.

α-(α-*Thymidyl*)*thymidine* **3**.—The FAB mass spectrum indicated that the molecule is a dimer; with a mass of 482 amu the molecule has two hydrogen atoms less than that of a true thymidine dimer. The ^1H NMR spectrum showed one set of magnetically distinct nucleosidic signals, indicating that there is a plane of symmetry in the molecule (Table 1). The evidence for the $-CH_2CH_2-$ linkage between the two base rings was fairly clear from the NMR spectrum. We noted the lack of any methyl signals; instead we detected a singlet corresponding to four protons, if we accept that the molecule is a dimer, at a chemical shift of δ 2.57 and in the same region as the methylene-bridge signals of 5-(α-thymidyl)-5,6-dihydrothymidine [dThd(α-5)-hdThd] and dThd(α-6)hdThd. Each nucleoside residue is unsaturated, which was clear from the downfield vinyl proton NMR signal and the absorption maximum in the UV spectrum of 272 nm.

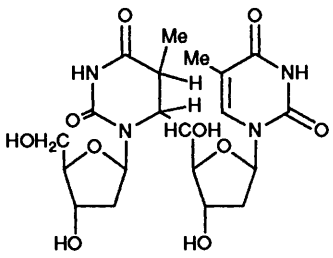
The only reasonable mechanism we may propose for the formation of α-(α-thymidyl)thymidine [dThd(α-α)dThd] is the direct combination of two $dThd^{\alpha}$ radicals. The fact that this product is formed in such significant yields compared with certain monomeric compounds[22] shows that deprotonation of the thymidine radical cation at the methyl group must be an important process, since it is statistically improbable that two $dThd^{\alpha}$ radicals form on adjacent molecules. This fact must also reflect the stability of the $dThd^{\alpha}$ radical. Indeed, in the solid state, the corresponding radical in γ-irradiated 1-methylthymine may be detected at temperatures as high as 200 °C.[32]

(5R*), *and* (5S*)-5-(*Thymidin*-3-*yl*)-5,6-*dihydrothymidine* **4a** *and* **4b**.—The yields of the two isomers of this product were considerably lower than those of the other adducts; however, the FAB mass spectrometric and ^1H NMR data appeared to be quite conclusive. FAB mass spectrometry gave the molecular weight at 484 amu, *i.e.*, a pure dimer, and the ^1H NMR spectra indicated two complete sets of osidic protons (Table 1). One vinyl proton signal was detected at δ *ca.* 8.0 as a tight quartet through long-range coupling to the methyl group whose signal was observed at δ 2.0. This rules out involvement of C(5), C(6), and the methyl group carbon in the linkage to the saturated pyrimidine moiety. Both osidic moieties are intact and hence the linkage does not involve any site within the osidic moiety either. The most likely alternative is the N(3) ring nitrogen. This was confirmed by the observation of only one imide proton signal when the spectrum was run in $(CD_3)_2SO$. The fact that the saturated base methyl signal is in the form of a singlet suggests that the linkage is to C(5). Its chemical shift at δ *ca.* 1.8 is higher than that for a C–C link. In addition, the signals assigned as the C(6) ring methylene protons are seen as a very wide AB system with one proton resonating at δ *ca.* 3.5 and the other at δ *ca.* 4.4. For the spore photoproducts and the 5-hydroxy-5,6-dihydrothymidines,[33] these signals are either degenerate or give a tight AB quartet. The NMR spectrum of one published adduct to thymidine at the 5-position shows similar base proton characteristics. That of the nitrogen-bound C(5) adduct of 4-*N*-oxyl-2,2,6,6-tetramethylpiperidone, a known radiosensitiser, also exhibits a higher than expected chemical shift for the methyl signal and highly magnetically non-equivalent C(6) methylene protons.[33]

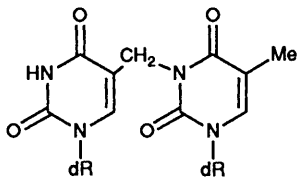
The proposed structure allows the possibility of two diastereoisomers with different configurations at C(5). The most likely mechanism for its formation is combination of an hdThd-5 radical with a dThd-3 radical.

6-(*Thymidin*-5′-*yl*)-5,6-*dihydrothymidine* **5a** *and* **5b**.—Although there are three asymmetric centres in this molecule, we have only isolated two of the possible eight diastereoisomers. The 400 MHz ^1H NMR data are listed in Table 1. The spectrum of each showed one downfield vinyl proton signal seen as a tight quartet through coupling to the methyl group protons whose signal was observed at δ *ca.* 1.9. The molecule evidently has one saturated and one unsaturated base residue.

The evidence for the site of the internucleoside bridge comes from an analysis of the furanosidic protons. The presence of a C(5) proton and only one (6)H proton signal at relatively low field at δ *ca.* 4 points to the presence of a bridge to C(6) of the saturated residue. We noted one complete set of osidic proton signals having the usual chemical shifts and coupling patterns for 2′-deoxyribonucleosides. It was evident, however, from the NMR spectrum that the other nucleoside residue lacks one of the geminal protons which usually constitute the exocyclic hydroxymethyl group. The (5′)H proton gives rise to a pseudotriplet in the spectrum of compound **5a** at δ 4.29 and a double doublet in that of compound **5b** at δ 3.82. The two-dimensional correlation spectra showed these protons to be coupled to protons having resonance signals at δ 3.82 and 3.98, respectively, which are themselves coupled to the (5)H protons and which must therefore be designated as (6)H. These observations are all consistent with the proposal of a covalent link between C(5′) of the unsaturated nucleoside residue to C(6) of the saturated one.



5a, b



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The dThd-5′ radical is formed in good yield as inferred from the observation of relatively high yields of the 5,6-dihydro-5′,6-cyclothymidines.[23] The 5,6-dihydrothymidin-6-yl radical which is proposed as a precursor to dThd(α-6)hdThd is likely to combine with the dThd-5′ radical leading to the formation of 6-(thymidin-5′-yl)-5,6-dihydrothymidine[dThd(5′-6)hdThd].

3-(α-*Thymidyl*)*thymidine* **6**.—The structural assignment for this compound has not been made unambiguously; however, the spectroscopic data suggest the title dThd(α-3)dThd structure. The molecular weight obtained from FAB mass spectrometry is 482 amu, consistent with the structure proposed. There are two complete independent sets of osidic proton signals in the 400 MHz ^1H NMR spectrum (Fig. 2),

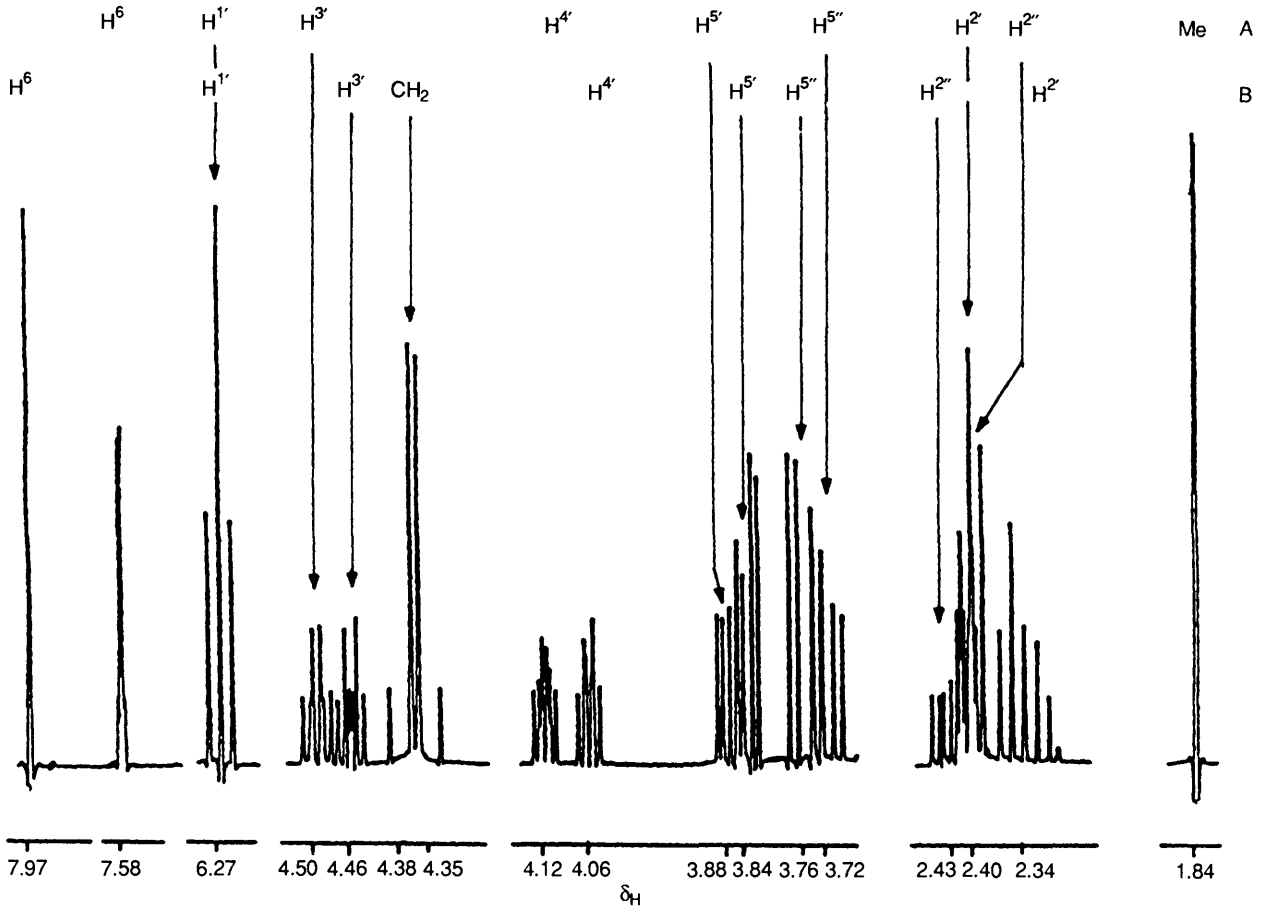

Fig. 2 400 MHz ^{1}H NMR spectrum of compound **6** recorded in D_2O *vs.* TSP internal standard

suggesting that the internucleoside linkage must take place between the two base moieties. It is interesting to note that the molecule has two vinyl protons. That seen at δ 7.58 represents a tight quartet indicating that it is coupled with the methyl group whose corresponding doublet signal is observed at δ 1.84. This indicates that the 5,6-double bond regions of one of the residues is intact, consistent with the involvement of N(3) in the internucleoside linkage. A well resolved spectrum indicated that the second vinyl proton signal is a tight triplet and is coupled to the methylene protons whose signals are seen as primarily an AB quartet at δ *ca.* 4.3. This may be interpreted as a methylene bridge to the N(3) position of the other nucleoside residue. This also explains the downfield shift of the methylene protons compared with those involved in a bridge to a carbon atom, such as in the spore photoproducts and the dThd(α-6)hdThd adducts. A factor which throws a slight doubt on our structural assignment is the observation of two broad signals in the region of the imide proton resonances when the ^{1}H NMR spectrum was run in [$^2H_6$]acetone; we believe this to be an artifact.

Assuming our assignment to be correct, this constitutes further proof of the deprotonation of the thymidine radical cation at the N(3) position since the likely mechanism of the formation of this product is combination of the resulting dThd-3 radical with a dThd$^\alpha$ radical.

Experimental

Materials and Equipment.—Nucleosides were obtained from the Pharma-Waldorf GmbH company and used without further purification. Perdeuteriated paraformaldehyde and deuteriated solvents for NMR were obtained from the Service des Molécules Marquées, Centre d'Etudes Nucléaires, Saclay, Paris.

Analytical HPLC separations were carried out using a Waters Associates M6000 pump equipped with a Waters Associates R401 refractometer and either a self-packed reversed-phase column (Macherey-Nagel Nucleosil 10 μm) or a self-packed silica gel column (Whatman Partisil 10 μm). Preparative HPLC separations were carried out on a Waters Associates LC/500 preparative HPLC apparatus equipped with a reversed-phase Prep Pak 500/C18 column (5.7 cm diameter, 30 cm long) and a refractive-index detector. Irradiations were effected using a ^{60}Co gamma source with an output of 187 Gy min^{-1}.

^{1}H and ^{13}C NMR spectra were obtained on a Bruker AM400 spectrometer with an Aspect 3000 computer. For ^{1}H NMR, samples were run in D_2O with sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate (TSP) as internal reference. For ^{13}C NMR, samples were run in D_2O and referenced against $SiMe_4$ external standard. Fast Atom Bombardment (FAB) mass spectrometric measurements were carried out using a GEC-AEI MS-50 apparatus, the sample presented as a glycerol mull under a bombardment of 5–8 keV argon atoms. The data were treated with an IBM 360-50 computer.

[CD_3]Thymidine was prepared in the following manner. A solution of 2'-deoxyuridine (3.42 g, 0.015 mol), perdeuteriated paraformaldehyde (6.0 g, 0.18 mol), NaOD (1 mol dm^{-3} in D_2O; 15 cm^3), and D_2O (15 cm^3) was boiled under reflux for eight hours. The reaction was controlled by analytical silica gel HPLC with ethyl acetate–propan-2-ol–water (75:16:9) as eluant. The reaction was halted by cooling in an ice-bath and neutralisation with Dowex 50W-X8 cation exchange resin. The residue was removed by filtration and the filtrate was

evaporated to dryness. By silica gel analytical HPLC using the above eluant, the yield of 5-hydroxymethyl-2′-deoxyuridine was estimated to be *ca.* 90%.

The reaction mixture obtained above (5.9 g) was dissolved in *ca.* 50% acetic [^{2}H]acid in D_2O prepared by addition of acetic anhydride (80 cm^3) to deuterium oxide (99.8% isotopic purity; 100 cm^3) and storage overnight. The resulting solution was transferred to a 1 dm^3 round-bottomed flask. In an efficient fume-cupboard, a rhodium–alumina catalyst (1.5 g) was added followed by deuterium gas, and the flask was stoppered and sealed with parafilm. The mixture was shaken mechanically for nine hours. Every hour the reaction mixture was analysed by reversed-phase analytical HPLC with water–methanol (9:1) as eluant and the atmosphere above the mixture was replenished in deuterium. Every three hours a further aliquot (1.5 g) of catalyst was added. The reaction mixture was finally filtered through a Millipore filter (0.45 μm) to remove the catalyst and the filtrate was evaporated to dryness. Residual acetic acid was removed by successive evaporations from ethanol. [CD_3]Thymidine was purified by preparative reversed-phase HPLC with water–methanol (9:1) as eluant. FAB mass spectrometry confirmed the molecular weight as 245 amu, and the ^{1}H NMR spectrum indicated no methyl-group resonance at the expected chemical shift. The deuteriothymidine was prepared in 45% yield with respect to the starting quantity of 2′-deoxyuridine.

Methodology.—Irradiation of thymidine. Thymidine (10.89 g) was dissolved in doubly distilled water (pH 6.5; 150 cm^3; 0.3 mol dm^{-3}) in a 250 cm^3 cylindrical flask with gentle warming and the resulting solution was deaerated by passage of nitrogen gas for 10 min. The flask was then sealed and the solution was rapidly frozen in liquid nitrogen. The sample was packed in solid CO_2 and irradiated at 196 K for 384 h (total dose 4.3 MGy). The sample was then allowed to anneal to room temperature and was then evaporated to dryness on a rotary evaporator. The bulk of the undegraded thymidine was removed by successive crystallisations and filtrations from hot ethanol down to a final filtrate volume of 20 cm^3. The filtrate was evaporated to dryness and the residue was taken up in water–methanol (9:1) (50 cm^3) and chromatographed by preparative reversed-phase HPLC column with water–methanol (9:1) as eluant. After elution of the residual thymidine, the column was washed with methanol and the washings were collected separately. These were evaporated to dryness and the resulting mixture was resolved in the following manner.

The mixture was firstly analysed by reversed-phase analytical HPLC with water–methanol (95:5) whereupon four peaks were observed after which the column was washed with methanol. All silica gel HPLC separations involved the use of ethyl acetate–propan-2-ol–water (75:16:9) as eluant. The first peak was shown to be residual thymidine and the second was *trans/anti*-cyclobutadithymidine.[22]

6-(α-*Thymidyl*)-5,6-*dihydrothymidine* **1a**. The third peak (k'_{rp} 11.2) was collected and evaporated to dryness to yield a white amorphous solid (22 mg), δ_C(external TMS, D_2O) 139.8 (d, C-6_u), 111.0 (s, C-5_u), 88.8 (d, C-4′$_u$), 87.8 (d, C-4′$_s$), 86.6 (d, C-1′$_s$), 85.4 (d, C-1′$_u$), 72.3 (d, C-3′$_u$), 71.6 (d, C-3′$_s$), 63.1 (d, C-5′$_u$), 62.9 (d, C-5′$_s$), 53.1 (d, C-6_s), 40.9 (d, C-5_s), 40.9 (t, C-2′$_u$), 40.2 (t, C-2′$_s$), 26.8 (t, CH_{2u}), and 11.3 (q, Me_s); λ_{max}(water) 262 nm; FAB-MS: *m/z* 507 ($M + Na^+$), 485 (MH^+), 369 ($MH^+ - dR$), 243 (dThd + H^+), 127 (BH^+), and 117 (dRH^+).

6-(α-*Thymidyl*)-5,6-*dihydrothymidine* **1b**. The fourth peak (k_{rp} 18.2) was collected, evaporated to dryness, and reinjected onto an analytical silica gel HPLC column, whereupon two further peaks were obtained by further elution. The first (k'_{Si} 8.33) was evaporated to dryness and yielded compound **1b** (11 mg), δ_C(external TMS, D_2O) 140.0 (d, C-6_u, 110.3 (s, C-5_u), 89.9 (d, C-1′$_s$), 88.8 (d, C-4′$_u$), 87.7 (d, C-4′$_s$), 85.9 (d, C-1′$_u$), 72.8 (d, C-3′$_s$), 72.1 (d, C-3′$_u$), 63.6 (d, C-5′$_s$), 62.8 (d, C-5′$_u$), 55.6 (d, C-6_s), 41.4 (d, C-5_s), 41.4 (t, C-2′$_u$), 38.2 (t, C-2′$_s$), 27.2 (t, CH_{2u}), and 11.3 (q, Me_s); λ_{max}(water) 262 nm; FAB-MS: *m/z* 507 ($M + Na^+$), 485 (MH^+), 369 ($MH^+ - dR$), 243 (dThd + H^+), 127 (BH^+), and 117 (dRH^+).

The second peak (k'_{Si} 11.3) gave 5-(α-thymidyl)-5,6-dihydrothymidine **2a** (3 mg), δ_C(external TMS, D_2O) 140.4 (d, C-6_u), 109.8 (s, C-5_u), 86.9 (d, C-4′$_u$), 85.9 (d, C-1′$_u$), 85.6 (d, C-4′$_s$), 84.2 (d, C-1′$_s$), 71.1 (d, C-3′$_s$), 70.6 (d, C-3′$_u$), 61.8 (d, C-5′$_s$), 61.3 (d, C-5′$_u$), 46.5 (t, C-6_s), 41.8 (s, C-5_s), 39.3 (t, C-2′$_u$), 35.5 (t, C-2′$_s$), 31.4 (t, CH_{2u}), and 19.7 (q, Me_s); λ_{max}(water) 270 nm; FAB-MS: *m/z* 507 ($M + Na^+$), 485 (MH^+), 369 ($MH^+ - dR$), 243 (dThd + H^+), 127 (BH^+), and 117 (dRH^+).

The methanol washings were evaporated to dryness and rechromatographed on a reversed-phase column with water–methanol (90:1) as eluant.

5-(α-*Thymidyl*)-5,6-*dihydrothymidine* **2b**. The first major peak (k'_{rp} 6.89) corresponded to compound **2b** (k'_{Si} 11.7, 3 mg), δ_C($SiMe_4$, D_2O) 140.5 (d, C-6_u), 109.9 (s, C-5_u), 86.8 (d, C-4′$_u$), 85.5 (d, C-4′$_s$), 85.5 (d, C-1′$_u$), 84.3 (d, C-1′$_s$), 71.1 (d, C-3′$_s$), 70.6 (d, C-3′$_u$), 61.9 (d, C-5′$_s$), 61.4 (d, C-5′$_u$), 47.0 (t, C-6_s), 41.8 (s, C-5_s), 38.9 (t, C-2′$_u$), 35.6 (t, C-2′$_s$), 31.7 (t, CH_{2u}), and 19.1 (q, Me_s); λ_{max}(water) 270 nm; FAB-MS: *m/z* 507 ($M + Na^+$), 485 (MH^+), 369 ($MH^+ - dR$), 243 (dThd + H^+), 127 (BH^+), and 117 (dRH^+).

5-(*Thymidin-3-yl*)-5,6-*dihydrothymidine* **4a**. The second peak (k'_{rp} 8.89) was rechromatographed on silica gel and yielded compound **4a** (k'_{Si} 11.67, 1 mg), FAB-MS: *m/z* 507 ($M + Na^+$) and 485 (MH^+).

5-(*Thymidin-3-yl*)-5,6-*dihydrothymidine* **4b**. The third peak (k'_{rp} 9.55) was also reinjected onto silica gel and yielded a peak (k'_{Si} 9.67) which, when evaporated to dryness, gave compound **4b** (1 mg).

6-(*Thymidin-5′-yl*)-5,6-*dihydrothymidine* **5a**. The peak eluting at k'_{rp} 11.44 was rechromatographed on silica gel to yield one major peak, corresponding to compound **5a** (k'_{Si} 5.50, 1 mg), FAB-MS: *m/z* 507 ($M + Na^+$) and 485 (MH^+).

α-(α-*Thymidyl*)*thymidine* **3**. The next peak (k'_{rp} 12.89) was evaporated to dryness, and rechromatographed on silica gel to give essentially one peak, corresponding to compound **3** (k'_{Si} 8.16, 5 mg), λ_{max}(water) 272 nm; FAB-MS: *m/z* 505 ($M + Na^+$) and 483 (MH^+).

6-(*Thymidin-5′-yl*)-5.6-*dihydrothymidine* **5b**. The next reversed-phase major peak at k'_{rp} 15.34 gave, after rechromatography on silica gel (k'_{Si} 6.78), compound **5b** (0.5 mg).

3-(α-*Thymidyl*)*thymidine* **6**. The last peak (k'_{rp} 21.67) was collected and again rechromatographed on silica gel. This gave one major peak (k'_{Si} 5.83), corresponding to compound **6** (2 mg), FAB-MS: *m/z* 505 ($M + Na^+$) and 483 (MH^+).

All compounds reported here appear extremely stable in the absence of extremes of temperature and pH. No decomposition was ever detected during their isolation and characterisation. Recrystallisation was attempted where sufficient quantities were available, but without success.

Conclusions

All of the products reported here, with the exception of the spore photoproducts (**2a** and **2b**) which result from a concerted mechanism linked to excitation processes, may be accounted for in terms of simple radical-combination processes. This represents good complementary evidence to ESR data which have in some cases indicated the formation of the same radical species. In addition, we have compounds whose formation suggests the generation of radicals so far not detected by ESR or ENDOR techniques, namely the N(3)-centred radical arising

from the deprotonation of the thymidine radical cation at this position, and the hdThd-6 radical formed apparently by hydrogen-atom transfer to C(5). The formation of the N(3)-centred radical appears to be a minor process compared with deprotonation of the radical cation at the methyl group. The dThd$^{\alpha}$ radical is implicated in the formation of several quantitatively important dimeric compounds, as well as in the formation of 5-hydroxymethyl-2′-deoxyuridine.[22] The deprotonation at N(3) leads to the formation of two quantitatively minor dimeric compounds

In the case of the spore photoproducts, we were somewhat surprised to note that the mechanism appeared to be entirely concerted and that no evidence was found for the combination of the dThd$^{\alpha}$ and the dThd-5 radicals, both of which were known to be formed in relatively high yields. The reason may well be that in cases where the oxidising potentials of the radicals concerned differ greatly (the dThd-5 radical is oxidising and the dThd$^{\alpha}$ radical may be reducing), electron transfer occurs rather than combination. For combination, the oxidising potentials of the radicals involved must not be too dissimilar.

Statistically the formation of two adjacent radicals is unlikely, hence the yields of certain radical combination products is low. This does not, however, mean that these products are likely to be biologically unimportant since it is not the yield of a particular lesion so much as its repairability which governs biological significance. Also, the possible formation of clusters of radiation damage sites in DNA, called spurs,[34] may increase the likelihood of formation of some of these new lesions. It is noteworthy that none of the products reported here may be directly linked to the involvement of water radicals, a fact which provides support for the use of the frozen aqueous medium for the study of the direct effects of ionising radiations on nucleic acid systems.

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