γ-Radiolysis and Hydroxyl Radical Produce Interstrand Cross-Links in DNA Involving Thymidine†

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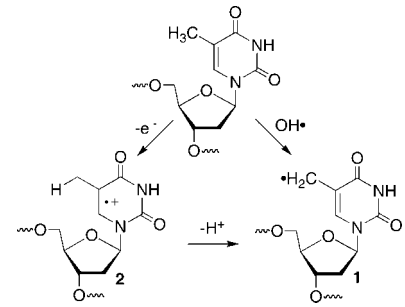
Interstrand cross-links are minor components of the collection of products formed in DNA by ionizing radiation. Through their formation by other damaging agents, it is known that interstrand cross-links exert significant effects on replication and transcription. The structures of DNA interstrand cross-links produced as a result of γ-radiolysis are unknown. Using synthetic duplexes we found that interstrand cross-link formation required thymidine and occurred with *G* values of $\sim 10^{-4}$ nmol J^{-1}. Enzymatic digestion of a tritiated substrate indicated that interstrand cross-links were derived from the reaction of 5-(2′-deoxyuridinyl)methyl radical (**1**) with the opposing 2′-deoxyadenosine to yield **5**, which was identical to the product previously characterized when **1** was independently generated from a synthetic precursor. Conservative estimates indicated that **5** accounted for at least one-fourth of the interstrand cross-links produced in DNA by γ-radiolysis. Utilization of a probe designed specifically to detect hole migration suggested that ~20% of the interstrand cross-links were produced by γ-radiolysis via this pathway. Experiments using an independent source of hydroxyl radical indicated that cross-links were also produced by this species. Hence, DNA interstrand cross-links arising from **1** should result from a variety of oxidative stress mechanisms.

Introduction

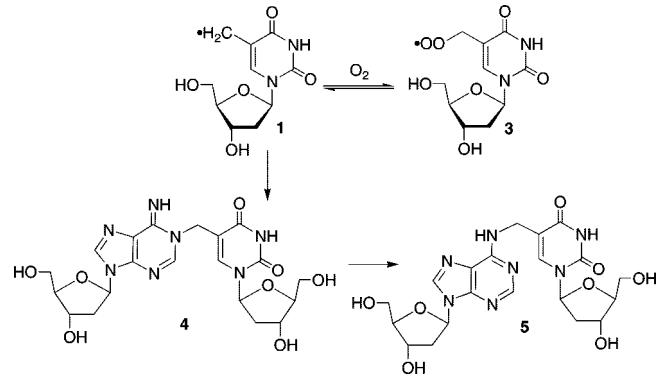
Exposing DNA to γ-radiolysis and other genotoxic agents produces a variety of types of DNA damage, including damaged nucleotides, single-strand breaks, double-strand breaks, and interstrand cross-links (ISCs). ISCs are formed in the smallest amounts and have not been well characterized. Even the most comprehensive, up-to-date treatise on DNA damage (*1*) provides little information on the yield (*G* value) or structure of ISCs produced via γ-radiolysis. Despite their low levels in DNA following oxidation, ISCs are important lesions because they prohibit replication and transcription if not repaired (*2–4*). Using a precursor capable of independently generating 5-(2′-deoxyuridinyl)methyl radical (**1**), we showed that this reactive intermediate, produced by formal hydrogen-atom abstraction from the methyl group of thymidine (dT), yields interstrand cross-links in good yield (*5, 6*). Experiments described herein have revealed that **1** is produced in DNA by γ-radiolysis and is a significant contributor to ISC formation.

It has been proposed (*1*) that **1** is formed in as much as 10% of the reactions between thymine and hydroxyl radical (Scheme 1). Deprotonation of the cation radical **2** also produces **1** (*7, 8*). A recent study proposed that this pathway plays a role in hole migration (electron transfer) in DNA, although ISCs were not reported (*9*). The fact that the hole migration studies were carried out under aerobic conditions should not have affected ISC formation via **1**, because studies in which **1** was independently generated indicated that cross-link formation involving **1** was independent of O_2 (*5, 6*). This unusual observation was explained by kinetic experiments on monomeric **1**, which showed that formation of peroxyl radical (**3**) was reversible (Scheme 2). In DNA, reaction of the alkyl radical **1** with the opposing 2′-deoxyadenosine (dA) to produce **5** (via **4**) is fast compared to the reactions of **3**. With the exception of the recent electron transfer experiments, independent studies of the reactivity and random generation of **1** suggest that γ-radiolysis should produce ISCs if this radical is indeed generated.

Scheme 1



Scheme 2



† Dedicated to Professor Larry Marnett on the occasion of his 60th birthday.

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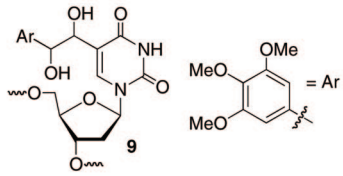
Chart 1. Sequences and/or Structures of Reagents Used in This Work

5′-d(TCA GTC AAT TTT ACT GAC)
3′-d(AGT CAG TTA AAA TGA CTG)
6

5′-d(AAC TGA TTA ACG T**X**G CAT TGT CAA TTT)
3′-d(TTG ACT AAT TGC AAC GTA ACA GTT AAA)
7 X = T
8 X = **9**



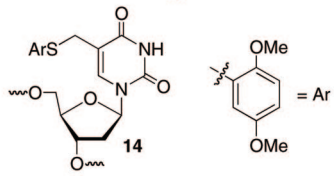
9

5′-d(GGC TCA CTC AAT TCA GTC AAT TTT ACT GAC TTT ACT GAC GCG)
3′-d(CCG AGT GAG TTA AGT CAG TTA AAA TGA CTG AAA TGA CTG CGC)
10

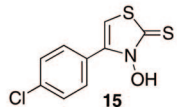
5′-d(GGC UCA CUC AAU UCA GUC AAU UUU ACU GAC UUU ACU GAC GCG)
3′-d(CCG AGU GAG UUA AGU CAG UUA AAA UGA CUG AAA UGA CUG CGC)
11

5′-d(GGC TCA CTC AAT TCA GTC AAT TTT ACT GAC TTT ACT GAC GCG)
3′-d(CCG AGT GAG TTA AGT CAG TTA AAA TGA CTG AAA TGA CTG CGC)
12 T = ^{3}H-thymidine

5′-d(AGA TGG AC**14** CAG GTA C)
3′-d(TCT ACC TG A GTC CAT G)
13



14



15

Materials and Methods

General Methods. [*methyl*,1′,2′-^{3}H]dTTP and [*methyl*-^{3}H]dTTP ammonium salts and [γ-^{32}P]ATP were purchased from Amersham. Snake venom phosphodiesterase (100 units, lyophilized powder) was purchased from USB. Calf intestinal alkaline phosphatase and Taq DNA polymerase were purchased from New England BioLabs. Oligonucleotides were synthesized via standard automated DNA synthesis on an Applied Biosystems model 394 instrument. Oligonucleotides containing **9** or **14** were prepared previously (*6, 10*). Radiolabeling was carried out using standard protocols (*11*). Radiolabeled oligonucleotides were hybridized with 1.5 equiv of complementary oligonucleotides in 10 mM potassium phosphate (pH 7.2) and 100 mM NaCl at 90 °C for 5 min and cooled to room temperature. Tritiated samples were counted using a Beckman Coulter LS 6500 scintillation counter. All anaerobic reactions were carried out in sealed pyrex tubes, which were degassed and sealed using freeze–pump–thaw (three cycles, 3 min each) degassing techniques. Thiazolethione (**15**) was prepared according to a literature procedure (*12*). Sequences and/or structures of reagents used in this work are shown in Chart 1.

Preparation of the 42-Nucleotide Tritiated Duplex 12 by PCR. A solution of [*methyl*,1′,2′-^{3}H]dTTP ammonium salt (4 nmol, 500 μL in 1:1 EtOH–H_2O, 1 mCi/mL) was dried in an Eppendorf tube (1.7 mL) by directing a gentle stream of argon onto the surface of the solution at room temperature for ~30 min. The dried [*methyl*,1′,2′-^{3}H]dTTP was dissolved in water (30 μL) and transferred to an Eppendorf tube (0.65 mL) containing template (5 μL of a 20 nM solution), a mixture of primers 1 and 2 (5 μL of a solution containing each primer at 5 μM), deoxynucleotide triphosphates (4 μL each of 1 mM solutions of dATP, dCTP, and dGTP), Taq DNA polymerase (1 μL of a 5 units/μL solution), and Taq DNA polymerase buffer [5 μL of a solution containing 20 mM Tris, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100, pH 8.8]. The 42-nucleotide (nt) tritiated duplex was prepared via PCR amplification of the above solution. PCR was performed using the following conditions: 94 °C (30 s) for melting, 55 °C (30 s) for annealing, and 72 °C (1 min) for the polymerase reaction. After 20 repetitions of the cycle, the reaction solution was extracted with phenol (1 × 50 μL) and diluted to 500 μL with water. The DNA was separated by spinning the solution (100 min at 14000*g*) through a Microcon filter (YM 3). After the filter was washed with water (3 × 400 μL), the PCR product **12** was recovered by adding water (3 × 33.3 μL) to the reversed filter and spinning for 3 min at 1000*g*. The PCR yield was determined by counting ^{3}H in the product (1.08×10^{-5} Ci, 6.25 pmol; theoretical yield 4.38×10^{-5} Ci, 25 pmol). The quality of PCR product was determined by 12% nondenaturing PAGE analysis of the 5′-^{32}P-labeling of the filtered PCR product (0.3 pmol); the product was found by phosphorimager analysis to be >95% pure. The sequences of the two primers and the template were as follows: primer 1, 5′-d(GGC TCA CTC AAT TCA GTC AAT); primer 2, 5′-d(CGC GTC AGT AAA GTC AGT AAA); and template, 5′-d(GGC TCA CTC AAT TCA GTC AAT TTT ACT GAC TTT ACT GAC GCG).

Preparation of the Tritiated Single Strand Using Terminal Deoxynucleotidyl Transferase. A solution of [*methyl*-^{3}H]dTTP ammonium salt (0.6 nmol, 31.7 μL in 1:1 EtOH–H_2O, 1 mCi/mL) was dried in an Eppendorf tube by directing a gentle stream of argon onto the surface of the solution at room temperature for ~20 min. The dried [*methyl*-^{3}H]dTTP was dissolved in water (15.8 μL). Primer (1.2 μL of a 5 μM solution), deoxynucleotide triphosphates (30 μL of 20 μM solutions, 0.6 nmol each of dATP, dCTP, and dGTP), terminal deoxynucleotidyl transferase (1 μL of a 20 units/μL solution), 10× NEB buffer 4 (6 μL, 500 mM potassium acetate, 200 mM Tris acetate, 100 mM magnesium acetate, and 10 mM dithiothreitol, pH 7.9), and 6 μL of 2.5 mM $CoCl_2$ were added to the solution of tritiated dTTP. The solution was incubated at 37 °C for 15 min and then heated at 70 °C for 10 min to inactivate the enzyme. The reaction solution was diluted to 500 μL with water and purified by centrifugation (100 min at 14000*g*) through a Microcon filter (YM 3). Water (2 × 200 μL) was passed through the filter to wash the single-strand product. The filter was reversed, and the tritiated material was recovered by adding water (3 × 33.3 μL) and spinning for 3 min at 1000*g*. The specific activity of the product (0.4×10^{-6} Ci/pmol) was determined by counting ^{3}H in the product (2.35×10^{-6} Ci) and assuming 100% recovery of the original DNA (6 pmol). The size distribution of the single-stranded product was determined by 20% denaturing PAGE analysis of a ^{32}P-labeled aliquot (0.3 pmol). The sequence of the primer was 5′-d(GGC TCA CTC AAT TCA GTC AAT)-3′.

γ-Radiolysis. γ-Radiolysis of 5′-^{32}P-labeled DNA [15 nM DNA, 10 mM potassium phosphate (pH 7.2), and 100 mM NaCl, total volume 50 μL] was carried out in Pyrex tubes using a J. L. Shepherd Mark I ^{137}Cs irradiator having an output of 25 Gy/min. After exposure, samples were dried in a speed-vac evaporator, resuspended in formamide loading buffer, separated

by 20% denaturing PAGE analysis, and analyzed using a phosphorimager.

For samples of the tritiated duplex **12**, a cocktail (150 μL) was prepared by mixing a stock solution of the purified PCR product (50 μL, 62.5 nM, 5.4×10^{-6} Ci), 1 M NaCl (15 μL), 100 mM KH_2PO_4 (15 μL), and water (70 μL). The cocktail was distributed (3×50 μL) in Pyrex tubes and irradiated (500 Gy) under anaerobic conditions. After γ-radiolysis, the three samples were combined, dried using a speed-vac evaporator, and resuspended in water (30 μL).

Enzymatic Digestion of Oligonucleotides and Isolation of the Tritiated dA–dT Coupling Product by HPLC. Snake venom phosphodiesterase was reconstituted from lyophilized solid into 110 mM Tris (pH 8.9), 110 mM NaCl, 15 mM $MgCl_2$, and 50% glycerol (2.94 mL, 34 units/mL). The irradiated, resuspended 30 μL sample of **12** described above was diluted with 0.1 M Tris buffer (200 μL, pH 8.9), and snake venom phosphodiesterase (5 μL, 0.17 unit) was added. The sample was incubated at 37 °C for 1 h. Calf intestinal alkaline phosphatase (5 μL, 50 units) was added, and the sample was incubated at 37 °C for an additional 1 h. The reaction mixture was diluted to 430 μL with H_2O and passed through a Microcon filter (YM 10) by centrifugation at 14000*g*. The filter was washed with water (2×100 μL), and the combined filtrate was lyophilized. The sample (4.39×10^{-6} Ci) was resuspended in 180 μL of buffer [10 mM KH_2PO_4 (pH 7.2) and 100 mM NaCl]. A fraction of this solution (3.37×10^{-7} Ci) was mixed with independently prepared (*5*) dA–dT coupling product **5** (1.2 nmol) and dT (16.5 nmol) in buffer [10 mM KH_2PO_4 (pH 7.2) and 100 mM NaCl]. A total volume of 100 μL was injected and analyzed by HPLC [C18-Microsorb column (4.6 mm $\times$ 25 cm)] with UV detection at 260 nm. The peaks of interest were collected using a linear gradient of 2% to 20% acetonitrile in water over 25 min. The retention times were 13.5 min for dT (2.58×10^{-7} Ci) and 20.4 min for the dA–dT coupling product (7.0×10^{-10} Ci).

Thiazolethione Photoreactions. Photoreactions of the duplexes were carried out in Pyrex tubes in a Rayonet photoreactor fitted with 16 lamps having a maximum output at 350 nm. The samples (50 μL each, using 10% acetonitrile as a cosolvent) were prepared from stock solutions of DNA (500 nM), 100 mM potassium phosphate (pH 7.2), 1 M NaCl, and **15** (19 mM in acetonitrile). No **15** was added when preparing control samples. After reaction, samples were dried, resuspended in formamide loading buffer, and subjected to 20% denaturing PAGE analysis.

γ-Radiolysis of ISCs Produced by Photolysis of 13. Photolysis (350 nm, 5 min) of [5′-^{32}P]**13** [15 nM DNA, 10 mM potassium phosphate (pH 7.2), and 100 mM NaCl, total volume 2×250 μL] was carried out in Pyrex tubes in a Rayonet photoreactor fitted with 16 lamps having a maximum output at 350 nm. The amount of ISC production ($1.35 \pm 0.01\%$) was determined via phosphorimager analysis. The samples were redistributed (6×50 μL) in Pyrex tubes and irradiated (500 Gy) using a J. L. Shepherd Mark I ^{137}Cs irradiator under aerobic and anaerobic conditions. After ^{137}Cs exposure, the samples were dried in a speed-vac evaporator, resuspended in formamide loading buffer, separated by 20% denaturing PAGE analysis, and analyzed using a phosphorimager.

Results and Discussion

γ-Radiolysis of DNA Produces Interstrand Cross-Links. Interstrand cross-link formation was investigated in three randomly chosen ^{32}P-labeled duplexes of synthetic oligonucleotides. Exposure of degassed samples of **6**, **7**, or **10** (Chart 1) to ^{137}Cs (500 Gy) produced small but readily measurable amounts of ISCs that were readily discernible via denaturing gel electrophoresis (Table 1). Increasing the exposure of **10** to as much as 3000 Gy actually resulted in a decrease in the ISC yield as measured by denaturing PAGE (data not shown). This could be explained by the fact that increasing the dose increased the fraction of duplexes that incurred multiple damage events, causing some ISC products to undergo cleavage at nucleotides located between the radiolabel and the position of the ISC. Hence, the amount of ISCs observed by denaturing PAGE could decrease even as the amount of cross-linked nucleotides increased. Initially, this observation appeared inconsistent with the idea of ISC formation through **1** and/or other DNA radicals. However, we reasoned that increasing the dose also increased the probability that the cross-linked DNA product was itself damaged and destroyed. In addition, the yield for ISCs was even lower in the presence of O_2 than under anaerobic conditions (Table 1). At first glance, this would appear to be inconsistent with the known oxygen enhancement effect on DNA damage by γ-radiolysis (*1*). However, O_2 increases the efficiency with which γ-radiolysis damages all DNA. It is possible that the cross-linked material is also degraded at a higher rate under aerobic conditions. In order to investigate this possibility, we utilized **13**, which contains **14** (Chart 1), a photochemical precursor to **1**. As expected, photolysis (350 nm, 5 min) of [5′-^{32}P]**13** produced ISCs ($1.35 \pm 0.01\%$) (*5, 6*). The photolyzed duplex was then exposed to ^{137}Cs (500 Gy) under aerobic and anaerobic conditions. A modest increase in ISC formation (to $1.89 \pm 0.12\%$) was observed following irradiation under anaerobic conditions. Importantly, the amount of ISCs actually decreased to $0.98 \pm 0.05\%$ (three replicates) following γ-radiolysis in the presence of O_2. In addition, the overall amount of direct strand breaks in **13** was ~36% greater in the presence of O_2 than under degassed conditions. These observations are consistent with the proposal that the major reason for the inverse effect of O_2 on ISC formation is not that it is required for cross-linking, but rather that the increased efficiency of DNA damage in the presence of O_2 results in a greater rate of cross-link destruction. Furthermore, these data indicate that the *G* values (Table 1) for ISC formation represent lower limits for the yields of this process.

Table 1. ISC Yields Produced by γ-Radiolysis (500 Gy) in Synthetic Duplexes

		% ISC formation (no. of replicates)		$10^4 \times$ average *G* value (nmol J^{-1})	
duplex	trial	anaerobic	aerobic	anaerobic	aerobic
6	1	1.51 ± 0.23 (3)	0.53 ± 0.14 (3)	4.5	1.6
	2	1.46 ± 0.06 (3)	0.37 ± 0.01 (3)	4.4	1.1
7	1	1.14 ± 0.07 (3)	0.28 ± 0.04 (3)	3.4	0.8
	2	1.12 ± 0.11 (3)	0.26 ± 0.02 (3)	3.4	0.8
	3	1.29 ± 0.10 (10)	–	3.9	–
8	3	1.05 ± 0.18 (12)	–	3.2	–
10	1	1.48 ± 0.06 (3)	0.32 ± 0.04 (3)	4.4	1.0
	2	1.44 ± 0.08 (3)	0.26 ± 0.04 (3)	4.3	0.8
11	3	<0.1 (3)	not detected	–	–

Role of 5-(2′-Deoxyuridinyl)methyl Radical 1 in γ-Radiolysis-Mediated Cross-Linking. Since **1** is the only documented example of a DNA radical that forms interstrand cross-links (*5, 6*), we sought to determine its involvement in γ-radiolysis-mediated cross-linking. Irradiation of [5′-^{32}P]**11**, a duplex in which all of the thymidines are replaced with 2′-deoxyuridines, resulted in almost complete elimination of cross-linking (Table 1), suggesting that the majority of ISC products involve thymidine. More direct evidence for the involvement of **1** was obtained using the 42 nt tritiated duplex **12**, which contains tritium at the thymidines indicated in Chart

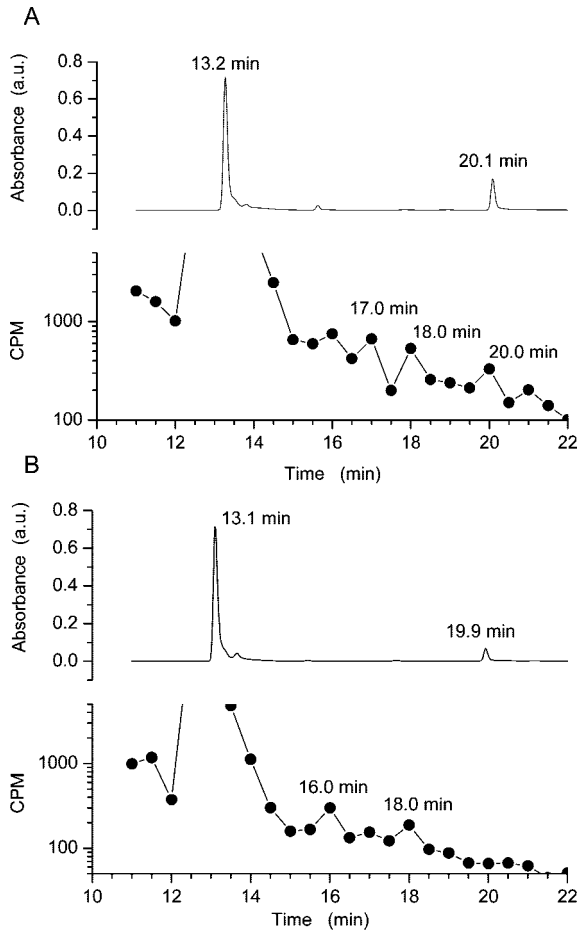
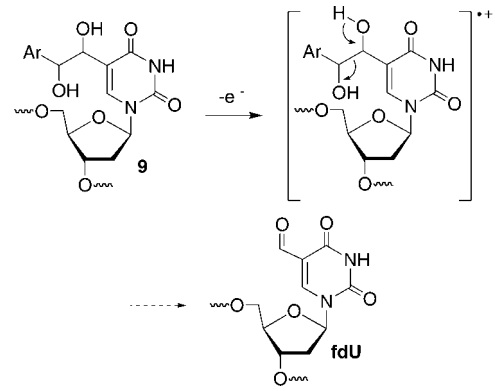


Figure 1. HPLC analysis (top) and tritium counting (bottom) of (A) enzymatically digested **12** and (B) single-stranded DNA following γ-radiolysis. The HPLC traces were spiked with thymidine (retention time 13.1–13.2 min) and **5** (retention time 19.9–20.1 min).

1. Tritium labeling enabled us to take advantage of the previous characterization of the dA–dT coupling product **5**, which is formed upon rearrangement of the initial cross-linked material (*5*). The tritiated duplex **12** was produced via PCR amplification using unlabeled 21 nt primers and commercially available [*methyl*,1′,2′-^{3}H]dTTP. Following anaerobic irradiation (500 Gy), the sample was enzymatically digested. A portion of the digested sample was spiked with thymidine and independently synthesized **5** and injected on a C_{18} reversed-phase HPLC column. Fractions were collected, and the tritium counts as a function of time were overlaid with the HPLC UV trace (Figure 1A). The peaks corresponding to thymidine and **5** were collected, and the amount of ^{3}H was determined by liquid scintillation counting. The analysis accounted for approximately 76% of the ^{3}H-labeled DNA subjected to digestion by the enzyme, and of this DNA, ~0.30% eluted along with **5**. The remainder of the ^{3}H was presumably associated with thymidine and other DNA lesions, some of which are apparent in the HPLC trace (Figure 1). The yield of **5** increased slightly (~0.42%) when the dose was increased to 3000 Gy. However, the yields were determined by measuring a small fraction of the total radiation and are not as accurate as those measured using sophisticated methods such as isotopic-dilution mass spectrometry.

Unlike other dimeric products, **5** has not been detected as a product of reaction of **1** with adjacent nucleotides. Nonetheless, the possibility that **5** arose from an intrastrand reaction was examined using single-stranded tritiated DNA as a substrate. Terminal deoxynucleotide transferase extension in the presence of tritiated thymidine triphosphate was used to prepare the tritiated substrate as a random mixture of oligonucleotides ranging from 30 to 100 nt in length (see Supporting Information). Importantly, enzymatic digestion revealed no evidence for **5** following irradiation of the single-stranded oligonucleotides containing tritiated thymidine (Figure 1B). The amount of radiation for which **5** accounted corresponded to approximately one-fourth the yield of cross-links typically detected by gel electrophoresis (Table 1). The fraction of the tritium that coeluted with **5** was independent of whether the sample was digested using snake venom phosphodiesterase and calf intestine alkaline phosphatase in either the presence or the absence of nuclease P1 and calf spleen phosphodiesterase (*13, 14*). This could indicate that other cross-linked products are formed or that our method lacks the quantitative accuracy of isotopic-dilution mass spectrometry (*15*). It is also possible that given the small amount of material produced, the actual amount of dA–dT coupling product **5** could be greater than that observed.

Scheme 3



What Pathways Are Responsible for Interstrand Cross-Links in DNA following γ-Radiolysis? Upon exposure to γ-radiolysis, DNA damage can occur via two general pathways (*1*). Direct ionization (the direct effect) produces cation radicals ("holes") that can migrate over long distances, but this effect occurs to only a very small extent because most of the energy is absorbed by H_2O (*16–18*). Ionization of H_2O (the indirect effect) produces a variety of reactive species that can damage DNA, including hydroxyl radical (OH•) and solvated electrons. The radical anions that result from excess electron addition to DNA are also believed to migrate through DNA, but the products ultimately derived from them are not as well characterized (*19–23*). Hydroxyl radical adds to the π bonds of nucleic-acid bases and abstracts hydrogen atoms, and the products derived from these reactions are well-studied (*1*). Product analysis is inadequate for determining the contributions of the direct and indirect effects of γ-radiolysis because common products are derived from the two pathways. For instance, holes produced by direct ionization react with water to produce the formal products of OH• addition (*24*). Cation radicals may also deprotonate to produce the formal products of hydrogen-atom abstraction (*8, 25*). Production of reactive intermediates formally attributable to the direct pathway that arise from initial damage by the indirect effect is evidenced by hole formation via β-phosphate cleavage in C4′ radicals that result from hydrogen-atom abstraction (*26, 27*).

We sought to determine the contribution of DNA holes to the formation of ISCs by γ-radiolysis using a mechanistic probe (**9**) that serves as an efficient trap of cation radicals and produces an alkali-labile lesion (**fdU**, Scheme 3) (*10*).

Table 2. ISC Yields Produced by Hydroxyl Radical in Synthetic Duplexes

	% ISC formation (no. of replicates)	
duplex	anaerobic	aerobic
7	0.43 ± 0.02 (3)	0.46 ± 0.03 (3)
8	0.46 ± 0.06 (3)	0.42 ± 0.01 (3)
10	0.35 ± 0.01 (2)	0.32 ± 0.04 (3)
10[a]	0.05 ± 0.01 (3)	0.03 ± 0.01 (3)

[a] With 10% 2-propanol added as an OH• scavenger.

Replacing a single thymidine in **7** with **9** (to give [5′-^{32}P]**8**) reduced the yield of ISCs by 18.5% (Table 1). Analysis of these data using an unpaired *t* test indicated a >99% probability ($p = 0.0015$) that the average amounts of ISCs were truly different and were attributable to a structural change associated with the incorporation of **9**. The observed effect of **9** on ISC formation indicated that the majority of the cross-links produced by γ-radiolysis resulted from direct hydrogen-atom abstraction from thymidine to produce **1** but that hole migration contributed to a minor extent. In contrast, no difference in the yield of ISCs (Table 2) was observed when [5′-^{32}P]**7**, [5′-^{32}P]**8**, or [5′-^{32}P]**10** was exposed to OH• produced via the photolysis of **15** (Chart 1). Furthermore, addition of 10% (v/v) 2-propanol to scavenge OH• significantly reduced the ISC yield, providing evidence for the formation of cross-links by this species. In addition, the ISC yield was independent of O_2 (Table 2), consistent with the role of 5-(2′-deoxyuridinyl)methyl radical **1** in cross-link formation.

Conclusions

These experiments have revealed that the majority of ISCs produced in DNA exposed to γ-radiolysis involve the reaction of thymidine with a nucleotide on the opposite strand. The efficiency of formation of ISCs was ~4 orders of magnitude lower than those of typical damaged nucleotides, such as 8-oxopurines, formamidopyrimidines, and thymidine glycol (*1*). A significant fraction of the ISCs resulted in the formation of the dA–dT coupling product **5** previously characterized in studies on the reactivity of the 5-(2′-deoxyuridinyl)methyl radical **1**. ISC formation was independent of O_2 when the DNA was damaged by independently generated OH•, consistent with the intermediacy of **1**. Utilization of a mechanistic probe for intercepting radical cations in DNA suggested that direct hydrogen-atom abstraction from thymidine to form **1** was the primary pathway responsible for ISC formation following γ-radiolysis. The ISCs resulting from holes may also have proceeded from **1** following deprotonation of the thymidine cation radical. Overall, these experiments confirm that 5-(2′-deoxyuridinyl) methyl radical **1** is produced by γ-radiolysis and results in formation of interstrand cross-links. In view of the general biological ramifications of interstrand cross-links, the consequences of these cross-links on DNA function should be evaluated (*4, 28–31*).

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Supporting Information Available: Autoradiogram of 5′-^{32}P-labeled single-stranded tritiated substrate. This material is available free of charge via the Internet at http://pubs.acs.org.

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