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# Complete release of (5′*S*)-8,5′-cyclo-2′-deoxyadenosine from dinucleotides, oligodeoxynucleotides and DNA, and direct comparison of its levels in cellular DNA with other oxidatively induced DNA lesions

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## ABSTRACT

**8,5′-Cyclopurine-2′-deoxynucleosides in DNA are repaired by nucleotide-excision repair, and act as strong blocks to DNA polymerases, RNA polymerase II and transcription factor binding. Thus, it is important to accurately determine the level of these lesions in DNA. There is controversy in the literature regarding the ability of different enzymes to release these compounds from oligodeoxynucleotides or DNA. We used liquid chromatography/mass spectrometry (LC/MS) to investigate the ability of several enzymes to release (5′*S*)-8,5′-cyclo-2′-deoxyadenosine [(5′*S*)-cdA] from dinucleotides and oligodeoxynucleotides and from DNA. The data show that (5′*S*)-cdA is completely released from DNA by hydrolysis with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase. The identity of the normal nucleoside 5′ to the (5′*S*)-cdA had a significant effect on its release. Using LC/MS, we also showed that the levels of (5′*S*)-cdA were within an order of magnitude of those of 8-hydroxy-2′-deoxyguanosine, and three times higher than those of 8-hydroxy-2′-deoxyadenosine in pig liver DNA. Different DNA isolation methods affected the levels of the latter two lesions, but did not influence those of (5′*S*)-cdA. We conclude that (5′*S*)-cdA can be completely released from DNA by enzymic hydrolysis, and the level of (5′*S*)-cdA in tissue DNA is comparable to those of other oxidatively induced DNA lesions.**

## INTRODUCTION

Cellular DNA constantly undergoes damage resulting from both endogenous and exogenous sources [reviewed in (1)]. Oxidative DNA damage in cells is caused by reactive oxygen species such as the hydroxyl radical (•OH) [reviewed in (2,3)]. The majority of oxidatively induced DNA base lesions in cells are repaired by the base-excision repair (BER) pathway [reviewed in (4)]. However, a specific class of DNA lesions called 8,5′-cyclopurine-2′-deoxynucleosides, i.e. 8,5′-cyclo-2′-deoxyadenosine (cdA) and 8,5′-cyclo-2′-deoxyguanosine (cdG), represents an exception to this general rule. These lesions result from the abstraction by •OH of an H atom from carbon-5′ of 2′-deoxyribose, followed by cyclization of the thus-formed sugar radical onto the carbon-8 position of the purine of the same nucleoside and, finally, oxidation of the resulting nucleobase radical [reviewed in (5)]. Both (5′*R*)- and (5′*S*)-diastereomers of cdA and cdG are formed. Oxygen inhibits this reaction due to its diffusion-controlled reaction with the sugar radical prior to cyclization. These compounds are tandem lesions because of the concomitant damage to both sugar and base moieties of the same nucleoside. The additional covalent bond between carbon-8 and carbon-5′ causes the DNA to assume an unusual structure (6,7), and also precludes repair of these lesions by a DNA glycosylase-initiated BER mechanism. As suggested previously (8,9), recent studies showed that 8,5′-cyclopurine-2′-nucleosides are not repaired by either BER or a direct repair mechanism, but are specifically repaired by the NER (10,11). This repair pathway is defective in the genetic disease xeroderma pigmentosum (XP) [reviewed in (12)]. (5′*S*)-cdA was shown to be a strong block to elongating DNA (11,13) and RNA (10) polymerases, and to block the binding of transcription factors to their cognate recognition sequences (14) (K.A Krone and P.J. Brooks, manuscript in preparation). These observations indicate that, if left unrepaired, cdA and, by analogy, cdG might have significant toxic effects in cells. Since NER represents the only known repair pathway for 8,5′-cyclopurine-2′-deoxynucleosides, these lesions may be responsible for some of the clinical abnormalities observed in XP or in other diseases that are unrelated to sun exposure, including neurodegeneration and an increased risk of certain types of internal cancers.

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The chemical nature of 8,5′-cyclopurine-2′-deoxynucleosides presents a unique analytical challenge. These compounds can only be released from DNA as free nucleosides by enzymic hydrolysis because of the presence of the covalent 8,5′-bond between the sugar and base moieties of the same nucleoside, excluding the use of acid hydrolysis. However, there is some controversy in the literature regarding the ability of different enzymes to release 8,5′-cyclopurine-2′-nucleosides from oligodeoxynucleotides or DNA. Previous studies used a combination of four enzymes, i.e. deoxyribonuclease I (or nuclease P1), snake venom phosphodiesterase, calf spleen phosphodiesterase and alkaline phosphatase (8,9,15–18). Released 8,5′-cyclopurine-2′-deoxynucleosides were detected using gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). Mass spectral behaviors of these compounds were elucidated for the electron-impact ionization mode in the case of GC/MS and for the atmospheric pressure ionization-electrospray process (API-ES) in the case of LC/MS. Background levels in cellular DNA were found to be quite low, but ionizing radiation and antitumor drug tirapazamine produced significant formation of both (5′*R*)- and (5′*S*)-diastereomers of cdA and cdG in DNA under anoxic conditions. These results clearly indicated that the aforementioned combination of enzymes were able to release 8,5′-cyclopurine-2′-deoxynucleosides from DNA. In contrast to these results, a recent paper claimed that free (5′*S*)-cdA could not be released from oligodeoxynucleotides containing this lesion using a similar combination of enzymes (19). No release of (5′*S*)-cdA at all was achieved when two oligodeoxynucleotides containing this lesion were hydrolyzed using a combination of nuclease P1 and bacterial alkaline phosphatase. Only trimers containing (5′*S*)-cdA were observed in addition to normal 2′-deoxynucleosides. Snake venom and calf spleen phosphodiesterases were not able to release (5′*S*)-cdA from the oligodeoxynucleotides, either (19). Finally, a recent publication using the $^{32}$P-postlabeling technique showed that the digestion of DNA with micrococcal nuclease plus spleen phosphodiesterase followed by a short treatment with nuclease P1 released dinucleotides containing a normal deoxynucleoside 5′ to cdA (20).

In view of these disparate results, we have now carried out a systematic analysis to investigate the ability of different enzymes to release free (5′*S*)-cdA from dinucleotides, oligodeoxynucleotides and DNA. Such information is crucial for the accurate measurement of the levels of 8,5′-cyclopurine-2′-deoxynucleosides under both basal conditions and in response to cellular stressors, such as drugs or ionizing radiation. We used LC/MS as the analytical technique since an LC/MS methodology for detecting both (5′*S*)-cdA and (5′*R*)-cdA had been established previously (16). In the present work, this technique was also found to be suitable for detecting dinucleoside monophosphates containing (5′*S*)-cdA that are the predicted products of incomplete enzymic hydrolysis.

## MATERIALS AND METHODS

### Materials

Nuclease P1 (from *Penicillium citrinum*) was purchased from Calbiochem, EMB Biosciences, Inc. (San Diego, CA). Calf thymus (ct)-DNA, snake venom phosphodiesterase and calf spleen phosphodiesterase were obtained from Sigma Chemical Co. (St Louis, MO). Alkaline phosphatase was purchased from Roche Diagnostics Corporation (Indianapolis, IN). 2′-Deoxyadenosine-5′-triphosphate-1,3,7,9-$^{15}N_4$-(4-amino-$^{15}$N) (dATP-$^{15}N_5$) and 2′-deoxyguanosine-5′-triphosphate-1,3,7,9-$^{15}N_4$-(2-amino-$^{15}$N) (dGTP-$^{15}N_5$) were from Medical Isotopes, Inc. (Pelham, NH). (5′*S*)-cdA was obtained from Berry & Associates, Inc. (Ann Arbor, MI). Acetonitrile (HPLC grade) was from Burdick and Jackson (Muskegon, MI). Biomax5 ultra filtration membranes (molecular mass cutoff of 5 kDa) were purchased from Millipore (Bedford, MA). Water (HPLC-grade) for LC/MS analyses was from J.T. Baker (Phillipsburg, NJ). Water purified through a Milli-Q system (Millipore) was used for all other applications. 2′-Deoxyadenylyl(3′→5′)-(5′*S*)-8,5′-cyclo-2′-deoxyadenosine 3′-monophosphate [d(AcA)-3′-p], 2′-deoxycytidylyl (3′→5′)-(5′*S*)-8,5′-cyclo-2′-deoxyadenosine 3′-monophosphate [d(CcA)-3′-p], 2′-deoxyguanylyl(3′→5′)-(5′*S*)-8,5′-cyclo-2′-deoxyadenosine 3′-monophosphate [d(GcA)-3′-p] and 2′-deoxythymidylyl(3′→5′)-(5′*S*)-8,5′-cyclo-2′-deoxyadenosine 3′-monophosphate [d(TcA)-3′-p], and oligodeoxynucleotides containing (5′*S*)-cdA and with no end phosphate groups were synthesized as described elsewhere (10,20).

### Irradiation of DNA

An aqueous buffered solution of DNA (10 mM phosphate buffer, pH 7.4, 0.3 mg/ml) was saturated with $N_2O$ and irradiated with gamma rays in a $^{60}$Co gamma-source at a dose of 2 Gy (dose rate 24 Gy/min). Subsequently, irradiated and unirradiated DNA solutions were dialyzed against water for 18 h. Water outside the dialysis tubes was changed three times during the course of dialysis. Aliquots of 50 μg of DNA were dried in a SpeedVac under vacuum.

### Isolation of DNA from pig liver

Frozen pig liver was obtained from Pel-Freeze, Inc. (Rogers, AK). Two methods of DNA isolation were used. The first method (phenol–chloroform method) was essentially as described previously (21). Briefly, tissues were homogenized in 10 vols of homogenizing buffer (1% SDS + 1 mM EDTA), then 500 μg/ml Proteinase K was added and incubated for 30 min at 37°C. The homogenates were then extracted with phenol, phenol–Sevag (chloroform/isoamyl alcohol in the ratio 24:1), and then with Sevag. The DNA was precipitated with 1 vol. of cold ethanol and dissolved in 0.01× SSC (1.5 mM NaCl, 0.15 mM sodium citrate, pH 8.0). The DNA was treated with RNase T1 (50 U/ml) and RNases A (50 μg/ml) for 30 min at 37°C, then extracted with equal volumes of Sevag. The DNA was precipitated from the aqueous layer using cold ethanol and washed with 70% ethanol. The DNA was then dissolved in 0.01× SSC at ∼1 mg/ml. We also tested the effect of adding 1.25 mM desferoximine (Sigma) to the homogenizing buffer prior to homogenization.

The second method was a high-salt extraction method as described previously (22,23). Frozen tissue (1 g) was homogenized in 8 ml Lysis solution (0.5 M Tris, pH 8.0, 20 mM EDTA, pH 8.0, 10 mM NaCl, 1% SDS). Proteinase K (0.5 mg/ml) was added to the mixture followed by incubation overnight at 37°C. The next day, 4 ml of saturated NaCl solution was

added to the mixture, which was vortex-mixed for 1 min, and then incubated at 56°C for 15 min. The mixture was centrifuged for 30 min with 16 000 *g* and the supernatant was collected. After another centrifugation, the DNA in the supernatant was precipitated using 1 vol. of cold ethanol, washed with 70% ethanol and dissolved in 0.01 × SSC. The DNA was then treated with RNases, extracted with Sevag, precipitated and dissolved as described above.

### Enzymic hydrolysis of dinucleotides, oligodeoxynucleotides and DNA

For enzymic hydrolysis, 2 pmol of (5′*S*)-cdA-$^{15}N_5$ as an internal standard was added to 100 pmol of dinucleotides and oligodeoxynucleotides. The amounts of (5′*S*)-cdA-$^{15}N_5$ added to 50 µg of unirradiated and irradiated ct-DNA samples were 0.2 and 1 pmol, respectively. After addition of the internal standard, dinucleotides, oligodeoxynucleotides and DNA samples were dried in a SpeedVac under vacuum, and then dissolved in 50 µl of 10 mM Tris–HCl solution (pH 7.5) supplemented with 2.5 µl of 1 M sodium acetate containing 45 mM $ZnCl_2$ (final pH 6.0). Aliquots of nuclease P1 (5 U), snake venom phosphodiesterase (0.004 U) and alkaline phosphatase (32 U) were added and the samples were incubated at 37°C for 6, 24 or 48 h. In some instances, spleen phosphodiesterase (0.004 U) was also used. The 3′-phosphate groups of dinucleotides were removed by hydrolysis with alkaline phosphatase for 6 h in the same manner to obtain dinucleoside monophosphates d(AcA), d(CcA), d(GcA) and d(TcA). After hydrolysis, the samples were filtered using ultrafiltration membranes with a molecular mass cutoff of 5 kDa by centrifugation at 6000 *g* for 30 min.

To determine whether the enzymic hydrolysis conditions used in the $^{32}$P-postlabeling studies reported previously (20) would also release (5′*S*)-cdA, 100 pmol of dinucleotides or oligodeoxydinucleotides were incubated in 10 µl of a solution containing 30 mM sodium succinate (pH 6), 10 mM $CaCl_2$, 500 mU micrococcal nuclease and 10 mU spleen phosphodiesterase (Worthington Biochemicals, Vineland, NJ). Digestion continued for 3.5 h at 37°C. Then, 4 µl of a solution containing 225 mM sodium acetate (pH 5.0), 0.55 mM $ZnCl_2$ and 5.6 µg nuclease P1 was added and incubation continued for 45 min at 37°C. The reaction was neutralized by adding 2.6 µl of 0.75 M CHES [2-(cyclohexylamino)ethanesulfonic acid] buffer (pH 9.7), and the samples were frozen on dry ice. The samples were subsequently thawed and treated with alkaline phosphatase as described above.

### Analysis by LC/MS

Analysis by LC/MS with the API-ES process using isotope-dilution technique and selected-ion monitoring (SIM) was performed as described previously (17). A Zorbax Eclipse XDB C18-reversed-phase column (15 cm × 2.1 mm i.d., 5 µm particle size) (Agilent Technologies, Rockville, MD) was used. A guard column packed with the same stationary phase (1 cm × 2.1 mm i.d.) was attached to the column head. The solvent A was a mixture of water and acetonitrile (98:2, v/v) and the solvent B was 100% acetonitrile. A gradient of 0.5% of the solvent B per minute was used. The flow rate was 0.2 ml/min. The column temperature was kept at 30°C. The isotopically labeled internal standard (5′*S*)-8,5′-cyclo-2′-deoxyadenosine-1,3,7,9-$^{15}N_4$-(4-amino-$^{15}$N) [(5′S)-cdA-$^{15}N_5$] was prepared using dATP-$^{15}N_5$ as described previously (18). Aliquots (20 µl) of filtered enzymic hydrolysates were injected onto the LC-column without any further treatment. When necessary, the effluents were passed through a UV-spectrophotometer for monitoring of the absorbance of (5′*S*)-cdA and dinucleoside monophosphates, before they were introduced into the ion chamber of the mass spectrometer. The identification and quantification of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) and 8-hydroxy-2′-deoxyadenosine (8-OH-dA) were performed as described elsewhere (24,25). The isotopically labeled internal standards 8-OH-dG-$^{15}N_5$ and 8-OH-dA-$^{15}N_5$ were isolated from gamma-irradiated solutions of dGTP-$^{15}N_5$ and dATP-$^{15}N_5$, respectively, using semi-preparative LC and the same procedure as described previously for the isolation of (5′*S*)-cdG-$^{15}N_5$ and (5′*S*)-cdA-$^{15}N_5$ (18).

## RESULTS

There are some discrepancies in the literature regarding the ability of different enzymes to release free (5′*S*)-cdA from DNA (see Introduction). To resolve these discrepancies, we used, in the present work, four dinucleotides consisting of each of the four normal 2′-deoxynucleosides 3′→5′-linked to (5′*S*)-cdA, and eight oligodeoxynucleotides with various sequences and a single (5′*S*)-cdA embedded at a defined position. Three other oligodeoxynucleotides with no (5′*S*)-cdA served as controls. The dinucleotides and the sequences of oligodeoxynucleotides used in the present work are shown in Table 1. Furthermore, unirradiated and irradiated DNA samples were used. All samples were hydrolyzed by a combination of nuclease P1, snake venom phosphodiesterase and alkaline phosphatase. In some instances, spleen phosphodiesterase was also used in addition to three enzymes to check whether it enhances the release of (5′*S*)-cdA. LC/MS was used for all measurements. The suitability of this technique for identification and quantification of (5′*S*)-cdA and (5′*R*)-cdA has been described

**Table 1.** Dinucleotides and the sequences of oligodeoxynucleotides used in this work

| | |
|---|---|
| d(AcA)-3′-p | |
| d(CcA)-3′-p | |
| d(GcA)-3′-p | |
| d(TcA)-3′-p | |
| 1. | 5′ d(GCATCTGTAAAAGCA*cA*TTGTTCCAGGAACC) 3′ |
| 2. | 5′ d(TCGAAATTAACCCTCACTAAAGGGAACAAA A*cA*GCTGGA) 3′ |
| 3. | 5′ d(AATTCCCGGGGATCCGTC*cA*ACCTGCAGCC AAGCT) 3′ |
| 4. | 5′ d(GAATTGTAATACGACTCACTATAGGGCG*cA* ATTGGGTAC) 3′ |
| 5. | 5′ d(CGAAACTGCTGG*cA*AGATTCCTGGC) 3′ |
| 6. | 5′ d(CATAGTTACAC*cA*TCTGCGAT) 3′ |
| 7. | 5′ d(CATAGTTACACGATCTGCGAT) 3′ [no (5′S) cdA] |
| 8. | 5′ d(TGGGAGGTCTA*cA*TAAGCAGAGCTCTCTGG) 3′ |
| 9. | 5′ d(TGGGAGGTCTATATAAGCAGAGCTCTCTGG) 3′ [no (5′S) cdA] |
| 10. | 5′ d(TCTGCTTAT*cA*TAGACCTCCCACCGTACACG) 3′ |
| 11. | 5′ d(TCTGCTTATATAGACCTCCCACCGTACACG) 3′ [no (5′S)-cdA] |

**Table 2.** The ions (*m/z*) and their masses (Da) of dinucleoside monophosphates obtained by LC/MS with API-ES in the positive-ionization mode

| | $MH^+$ | $MNa^+$ | $MNaNa^+$ |
|---|---|---|---|
| d(AcA) | 563 (100%) | 585 (80%) | 607 (75%) |
| d(CcA) | 539 (100%) | 561 (90%) | 583 (10%) |
| d(GcA) | 579 (10%) | 601 (100%) | 623 (60%) |
| d(TcA) | 554 (100%) | 576 (90%) | 598 (65%) |

Relative intensities are given in parentheses.

previously (16). The sensitivity level of LC/MS with SIM for detecting these compounds has been determined to be very high. An amount of 2 fmol of (5′*S*)-cdA eluting from the LC-column can readily be detected by the mass spectrometer with a signal-to-background ratio of ~4 (16).

### LC/MS of dinucleotides and (5′*S*)-cdA

First, we investigated whether LC/MS would be suitable to detect dinucleoside monophosphates, i.e. d(AcA), d(CcA), d(GcA) and d(TcA). This is because these compounds are likely products of putative incomplete hydrolysis of (5′*S*)-cdA-containing oligodeoxynucleosides and DNA, and are well-suited for testing the capability of the enzyme combination to hydrolyze the 3′→5′-phosphate bond between a normal nucleoside and (5′*S*)-cdA. First, 3′-phosphate groups of the four dinucleotides were removed by treatment with alkaline phosphatase. The resulting dinucleoside monophosphates were analyzed by LC/MS along with (5′*S*)-cdA to examine their elution behavior, and to determine their mass spectral characteristics and characteristic ions using the total-ion monitoring mode. Then, LC/MS with SIM was used to identify and quantify them in actual samples. The positive-ion API-ES mass spectra of all four dinucleoside monophosphates exhibited a protonated molecular ion ($MH^+$), a Na-adduct ion ($MNa^+$) and an adduct ion with two Na atoms ($MNaNa^+$). The intensities of these ions varied among the compounds. Table 2 shows the ions and their relative intensities. The positive-ion API-ES mass spectrum of (5′*S*)-cdA was published previously (16). The typical ions of this compound are a fragment ion at *m/z* 164 and the $MH^+$ at *m/z* 250. The former results from the simultaneous cleavage of the *N*-glycosidic bond and the bond between the 5′-carbon and 4′-carbon of the sugar moiety with an H atom transfer, and contains the base moiety and the 5′-CHOH portion of the sugar moiety plus an H atom (16). The masses of these ions are switched by 5 Da to 169 and 255 Da, respectively, in the mass spectrum of the internal standard (5′*S*)-cdA-$^{15}N_5$ (18). Figure 1 illustrates the elution order of dinucleoside monophosphates and (5′*S*)-cdA as analyzed by LC/MS with SIM using some of their characteristic ions. These compounds gave symmetrical and sharp signals indicating the suitability of LC/MS for their analysis.

### Enzymic digestibility of dinucleotides and oligodeoxynucleotides containing (5′*S*)-cdA

The hydrolysis time periods of 6, 24 and 48 h were tested to investigate the digestibility of dinucleotides, oligodeoxynucleotides and DNA containing (5′*S*)-cdA. After hydrolysis, the samples were analyzed by LC/MS with SIM by monitoring the typical ions of the dinucleoside monophosphates (Table 2),

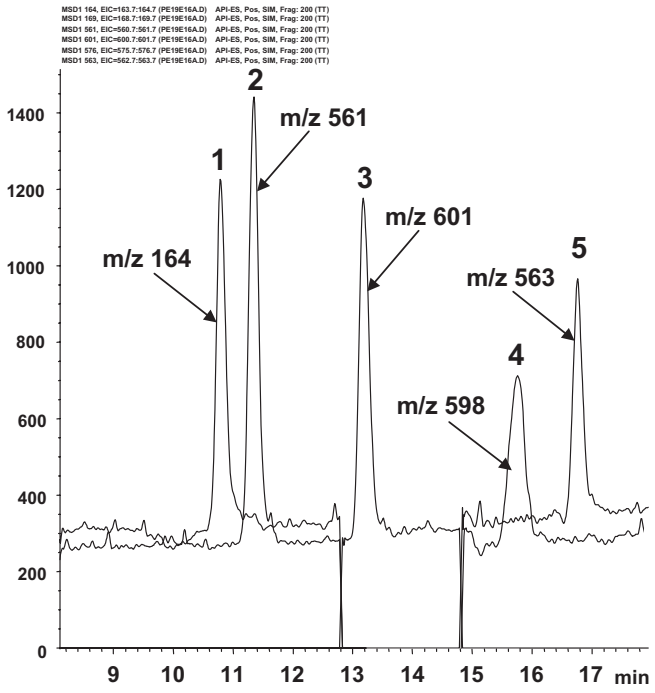


**Figure 1.** Ion–current profiles recorded during LC/MS-SIM analysis of a mixture of (5′*S*)-cdA and dinucleoside monophosphates. Peaks: 1, (5′*S*)-cdA; 2, d(CcA); 3, d(GcA); 4, d(TcA); and 5, d(AcA). See Table 2 for the characteristic ions.

(5′*S*)-cdA (*m/z* 164 and *m/z* 250) and (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169 and *m/z* 255) at appropriate retention time periods, where these compounds eluted from the LC-column. Furthermore, the UV-absorbance of the effluents was monitored. These two monitoring modes permitted to quantify the degree of hydrolysis in terms of the increase and decrease in the amounts of (5′*S*)-cdA and dinucleoside monophosphates, respectively. Figure 2 illustrates the results obtained with d(AcA)-3′-p after hydrolysis with nuclease P1, snake venom phosphodiesterase and alkaline phosphatase for 6, 24 and 48 h. The ion–current profiles of the characteristic ions of (5′*S*)-cdA (*m/z* 164), (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) and d(AcA) (*m/z* 563, *m/z* 585 and *m/z* 607) are shown. Figure 2A shows that d(AcA)-3′-p was clearly hydrolyzed to yield (5′*S*)-cdA, but some d(AcA) still remained after 6 h of hydrolysis. After 24 h (Figure 2B), the release of (5′*S*)-cdA increased judging from the ratio of *m/z* 164 [(5′*S*)-cdA] to *m/z* 169 [(5′*S*)-cdA-$^{15}N_5$]. The ions of d(AcA) and its UV-absorbance were still detectable, but to a much smaller extent than in Figure 2A. No d(AcA) was detectable after 48 h of hydrolysis, indicating the complete hydrolysis of d(AcA)-3′-p to give (5′*S*)-cdA and dA. The percentages of the release of (5′*S*)-cdA are given in Table 3. d(GcA)-3′-p was readily hydrolyzed to release (5′*S*)-cdA to the extent of 86% after 6 h (Table 3). Hydrolysis was completed after 24 h (chromatogram not shown). In contrast, d(TcA)-3′-p exhibited a greater resistance to hydrolysis. Figure 3 illustrates the ion–current profiles of (5′*S*)-cdA (*m/z* 164), (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) and d(TcA) (*m/z* 554, *m/z* 576 and *m/z* 598). The release of (5′*S*)-cdA by hydrolysis of d(TcA)-3′-p steadily increased with increased hydrolysis time (Figure 3A and B). However, no complete hydrolysis of d(TcA)-3′-p was observed (Figure 3C). Only

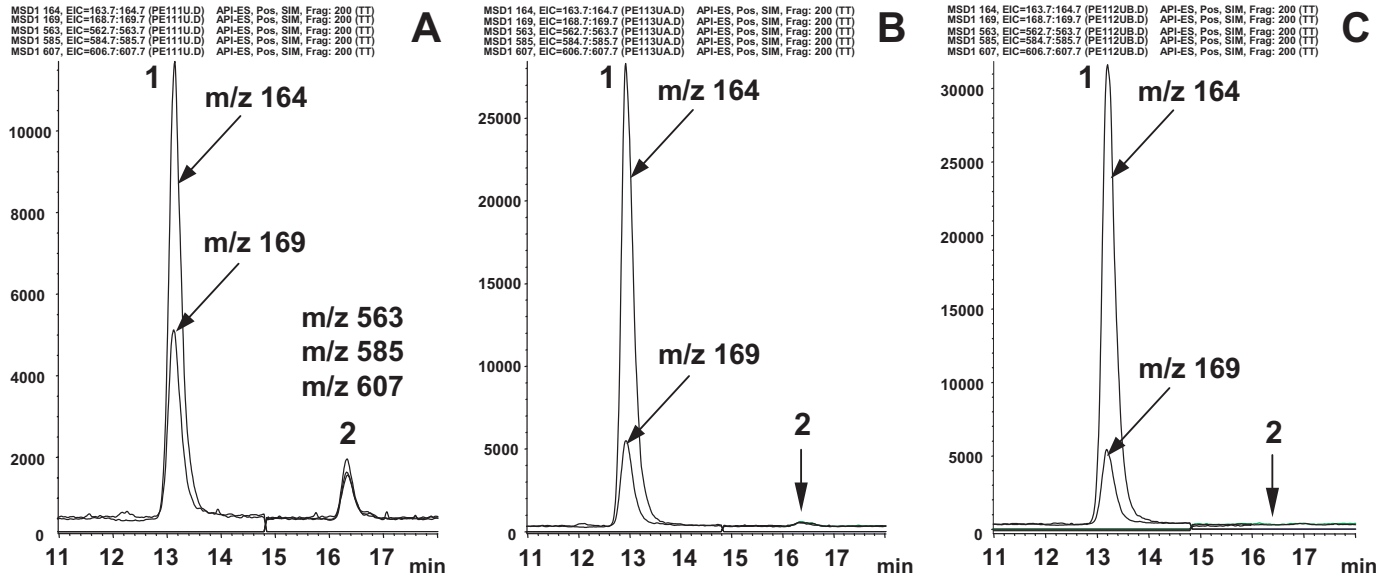


**Figure 2.** Ion–current profiles of the characteristic ions of (5′*S*)-cdA, (5′*S*)-cdA-$^{15}N_5$ and d(AcA) recorded during LC/MS-SIM analysis of the enzymic hydrolysate of d(AcA). A, B and C, hydrolysis for 6, 24 and 48 h, respectively. Peaks: 1, (5′*S*)-cdA (*m/z* 164) and (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) and 2, d(AcA).

**Table 3.** Percentage[a] of the release of (5′*S*)-cdA from dinucleotides and oligodeoxynucleotides by hydrolysis at different incubation time periods

| | 6 h | 24 h | 48 h |
|---|---|---|---|
| Dinucleotides | | | |
| d(AcA)-3′-p | 42.2 ± 1.7 | 91.1 ± 1.4 | 100[b] |
| d(GcA)-3′-p | 85.6 ± 1.5 | 100 | 100 |
| d(TcA)-3′-p | 9.77 ± 0.26 | 29.4 ± 0.7 | 47.5 ± 0.5 |
| d(CcA)-3′-p | 22.8 ± 0.7 | 76.3 ± 0.5 | 100 |
| Oligodeoxynucleotides | | | |
| 1 | 100 | 100 | 100 |
| 2 | 100 | 100 | 100 |
| 3 | 100 | 100 | 100 |
| 4 | 90.2 ± 2.1 | 100 | 100 |
| 5 | 86.7 ± 0.9 | 100 | 100 |
| 6 | 100 | 100 | 100 |
| 8 | 41.8 ± 2.8 | 78.1 ± 2.7 | 100 |
| 10 | 50.8 ± 2.6 | 89.4 ± 0.3 | 100 |

[a]Percentages were calculated on the basis of the UV-absorbance at 260 nm. The contribution of (5′*S*)-cdA-$^{15}N_5$ to the UV-absorbance of (5′*S*)-cdA was subtracted from the UV-signal on the basis of the ratio of the signal area of *m/z* 164 to that of *m/z* 169 as measured by LC/MS. The numbers represent the mean ± standard deviation from three independent measurements.
[b]100% means that no corresponding dinucleoside monophosphate was detectable by LC/MS and UV-absorbance, and the amount of released (5′*S*)-cdA did not increase after the corresponding incubation time.

~50% of (5′*S*)-cdA was released after 48 h of hydrolysis (Table 3). d(CcA)-3′-p was also somewhat resistant to hydrolysis; however, the release of (5′*S*)-cdA from this compound was greater than that from d(TcA)-3′-p (chromatogram not shown). The hydrolysis amounted to ~76% after 24 h, and was complete after 48 h (Table 3).

Oligodeoxynucleotides whose sequences are shown in Table 1 were hydrolyzed in the same manner as dinucleotides. The hydrolysis of oligodeoxynucleotides 1 and 2 containing the sequence d(AcA) and that of the oligodeoxynucleotide containing the sequence d(CcA) were complete after 6 h. This means that no d(AcA) or d(CcA) was detectable by LC/MS or UV-absorbance, and the amount of released (5′*S*)-cdA did not increase after 6 h of hydrolysis. The oligodeoxynucleotides 4, 5 and 6 containing the sequence d(GcA) were hydrolyzed to an extent of 87–100% after 6 h. The hydrolysis of 4 and 5 was complete after 24 h. On the other hand, the oligodeoxynucleotides 8 and 10 containing the sequence d(TcA) exhibited more resistant to hydrolysis. Figure 4 illustrates the ion–current profiles of (5′*S*)-cdA (*m/z* 164), (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) and d(TcA) (*m/z* 554, *m/z* 576 and *m/z* 598) obtained with the oligodeoxynucleotide 8. After 6 h, these oligomers were hydrolyzed to an extent of ~42 and 51%, respectively. The hydrolysis significantly increased after 24 h and reached completion after 48 h (Figure 4 and Table 3). The oligodeoxynucleotides 7, 9 and 11 without (5′*S*)-cdA were used as controls, and no (5′*S*)-cdA was detected.

## Enzymic digestibility of DNA containing (5′*S*)-cdA

Calf thymus DNA was hydrolyzed under the same conditions as dinucleotides and oligodeoxynucleotides and then analyzed by LC/MS to examine whether any dinucleotides would be detectable, possibly indicating an incomplete hydrolysis. In addition, ct-DNA was gamma-irradiated at a dose of 2 Gy, and then hydrolyzed and analyzed by LC/MS. In both instances, there was no increase in the level of (5′*S*)-cdA beyond 6 h of hydrolysis. No presence of d(AcA), d(GcA), d(TcA) or d(CcA) was observed, either, after 6, 24 or 48 h, indicating a complete release of (5′*S*)-cdA from DNA. Figure 5 illustrates the ion–current profiles of the characteristic ions of (5′*S*)-cdA, (5′*S*)-cdA-$^{15}N_5$ and d(TcA) obtained during LC/MS analysis of gamma-irradiated DNA hydrolyzed for 6 h. As can be seen, (5′*S*)-cdA was clearly detectable, whereas no signal for d(TcA) was observed (elution position indicated by the arrow). The levels of (5′*S*)-cdA measured in unirradiated and irradiated ct-DNA samples are given in Table 4, and clearly show no additional release of this compound from DNA beyond 6 h of hydrolysis.

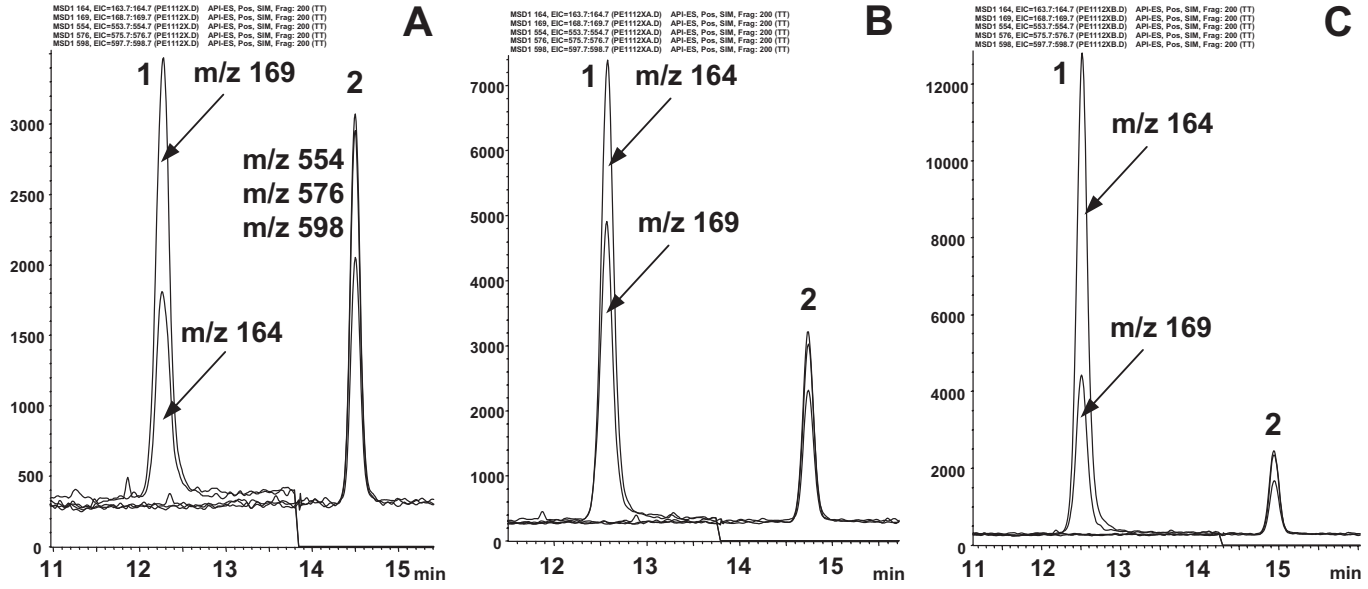


**Figure 3.** Ion–current profiles of the characteristic ions of (5′*S*)-cdA, (5′*S*)-cdA-$^{15}N_5$ and d(TcA) recorded during LC/MS-SIM analysis of the enzymic hydrolysate of d(TcA). A, B and C, hydrolysis for 6, 24 and 48 h, respectively. Peaks: 1, (5′S)-cdA (*m/z* 164) and (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) and 2, d(TcA).

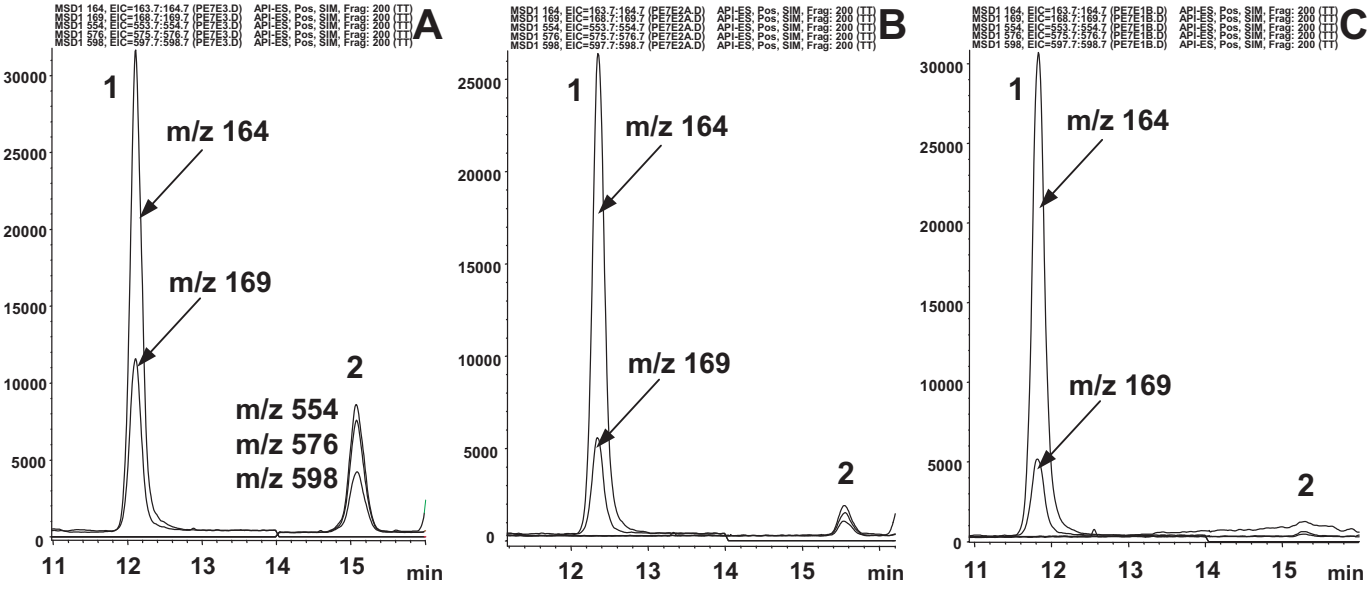


**Figure 4.** Ion–current profiles of the characteristic ions of (5′*S*)-cdA, (5′*S*)-cdA-$^{15}N_5$ and d(TcA) recorded during LC/MS-SIM analysis of the enzymic hydrolysate of oligodeoxynucleotide 8 (Table 1). A, B and C, hydrolysis for 6, 24 and 48 h, respectively. Peaks: 1, (5′*S*)-cdA (*m/z* 164) and (5′S)-cdA-$^{15}N_5$ (*m/z* 169); 2, d(TcA).

We also asked whether the enzymic hydrolysis conditions used in the previously reported $^{32}$P-postlabeling studies (20) would release (5′*S*)-cdA. Dinucleotides and oligodeoxynucleotides 2–4 and 11 (Table 1) were hydrolyzed under those conditions followed by additional hydrolysis with alkaline phosphatase. Small amounts of released (5′*S*)-cdA were observed. The extent of release varied among dinucleotides and oligodeoxynucleotides, and was much less than that observed with the other enzyme combination described above (Table 5). d(TcA)-3′-p and the oligodeoxynucleotide with sequence d(TcA) were the least digestible.

## Background levels of (5′*S*)-cdA and other lesions in cellular DNA

The background levels of (5′*S*)-cdA and, for comparison, those of 8-OH-dG and 8-OH-dA in genomic DNA of pig liver isolated by various methods and in ct-DNA were measured. Figure 6 illustrates an example of ion–current profiles of the characteristic ions of (5′*S*)-cdA (*m/z* 164) and (5′*S*)-cdA-$^{15}N_5$ (*m/z* 169) recorded during the analysis of a pig liver DNA sample by LC/MS-SIM. Table 6 shows the levels of (5′*S*)-cdA, 8-OH-dG and 8-OH-dA as measured after 24 h of enzymic

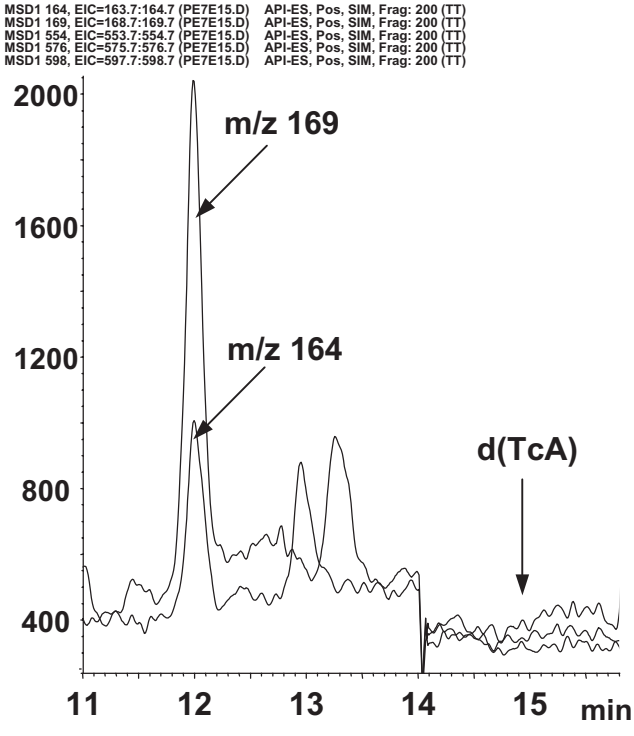


**Figure 5.** Ion–current profiles of the characteristic ions of (5′*S*)-cdA, (5′*S*)-cdA-$^{15}N_5$ and d(TcA) recorded during LC/MS-SIM analysis of the enzymic hydrolysate of ct-DNA irradiated in aqueous solution at 2 Gy. Hydrolysis time was 24 h.

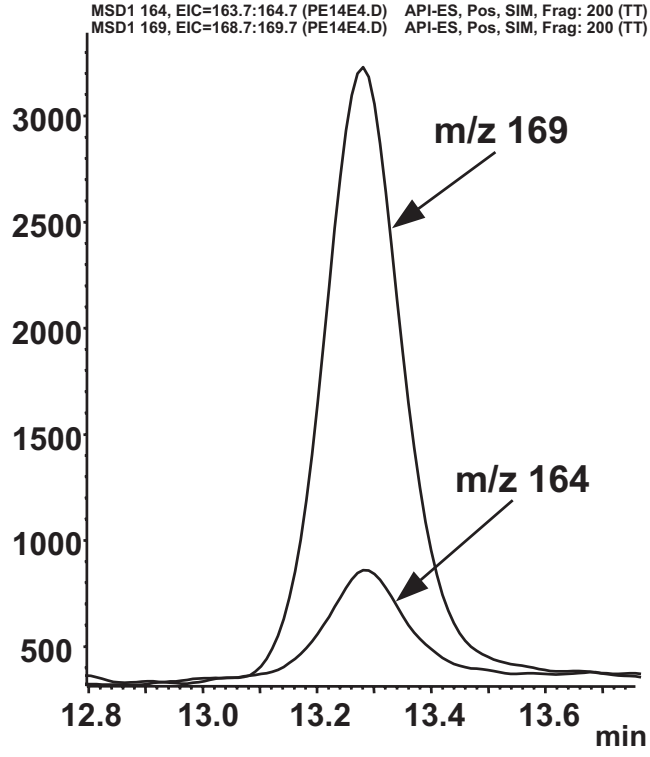


**Figure 6.** Ion–current profiles of the characteristic ions of (5′*S*)-cdA and (5′*S*)-cdA-$^{15}N_5$ recorded during LC/MS-SIM analysis of the enzymic hydrolysate of a pig liver DNA sample isolated by the high-salt extraction method. Hydrolysis time was 24 h.

**Table 4.** Levels (lesions/$10^6$ DNA bases)[a] of (5′*S*)-cdA released by hydrolysis at different incubation time periods from ct-DNA before and after gamma irradiation at 2 Gy

| ct-DNA | 6 h | 24 h | 48 h |
|---|---|---|---|
| Unirradiated | 0.698 ± 0.017[b] | 0.719 ± 0.027[b] | 0.723 ± 0.017[b] |
| Gamma irradiated at 2 Gy | 1.70 ± 0.04 | 1.65 ± 0.03 | 1.69 ± 0.09 |

[a]The numbers represent the mean ± standard deviation from three independent measurements.
[b]Statistically different from the value in line 2 ($P < 0.05$).

**Table 5.** Percentage[a] of the release of (5′*S*)-cdA from dinucleotides and oligodeoxynucleotides by hydrolysis under conditions described previously (20)

| | Percentage |
|---|---|
| Dinucleotides | |
| d(AcA)-3′-p | 15.3 ± 1.7 |
| d(GcA)-3′-p | 9.4 ± 4.3 |
| d(TcA)-3′-p | 3.8 ± 0.5 |
| d(CcA)-3′-p | 13.6 ± 0.7 |
| Oligodeoxynucleotides | |
| 2 | 9.0 ± 0.8 |
| 3 | 19.1 ± 1.6 |
| 4 | 14.3 ± 5.6 |
| 10 | 1.1 ± 0.1 |

[a]The numbers represent the mean ± standard deviation from three independent measurements.

**Table 6.** Levels (lesions/$10^6$ DNA bases)[a] of (5′*S*)-cdA, 8-OH-dG and 8-OH-dA released from pig liver DNA and from ct-DNA after hydrolysis for 24 h

| ct-DNA | (5′*S*)-cdA | 8-OH-dG | 8-OH-dA |
|---|---|---|---|
| pl-DNA, PC(1) | 0.706 ± 0.033 | 4.70 ± 0.17[b] | 0.202 ± 0.024[e] |
| pl-DNA, PC(2) | 0.723 ± 0.016 | 4.03 ± 0.26[c] | 0.176 ± 0.005[e] |
| pl-DNA, PC(1) + Des | 0.717 ± 0.003 | 3.48 ± 0.09[c] | 0.157 ± 0.033[e] |
| pl-DNA, HS | 0.744 ± 0.017 | 2.63 ± 0.14[d] | 0.191 ± 0.019[e] |
| ct-DNA | 0.719 ± 0.027 | 49.5 ± 1.5 | 1.85 ± 0.05 |

[a]The numbers represent the mean ± standard deviation from three independent measurements.
[b]Statistically different from the values in lines 3-5 ($P < 0.05$).
[c]Statistically different from the values in lines 4 and 5 ($P < 0.05$).
[d]Statistically different from the value in line 5 ($P < 0.05$).
[e]Statistically different from the value in line 5 ($P < 0.05$). pl-DNA, pig liver DNA; PC, phenol–chloroform method (21); PC + Des, phenol–chloroform method in which desferroximine was included in the homogenization mixture; HS, high-salt method (22,23). pl-DNA PC(1) and pl-DNA PC(1) + Des were processed together on the same day; pl-DNA PC(2) and pl-DNA HS were also processed together on a different day. All four pl-DNA samples were prepared using the same batch of frozen tissue that had been stored at −80°C.

hydrolysis. There was no statistical difference between the background levels of (5′*S*)-cdA in pig liver DNA samples isolated using various DNA isolation methods. Ct-DNA exhibited a similar background level of (5′*S*)-cdA to those observed in pig liver DNA. This indicates that different isolation methods did not affect the level of (5′*S*)-cdA. In contrast, the level of 8-OH-dG in DNA samples isolated from pig liver using a protocol involving phenol-chloroform (21) was significantly

greater than that found in DNA isolated by a method using high-salt extraction (22,23). The inclusion of desferroximine in the homogenizing buffer prior to homogenization significantly reduced the level of 8-OH-dG; however, the level observed was still statistically greater than that obtained using the high-salt extraction method. Ct-DNA had more than an order of magnitude greater level of 8-OH-dG than pig liver DNA. These results indicate the effect of DNA isolation method on the level of 8-OH-dG. The level of 8-OH-dA in ct-DNA was also about an order of magnitude greater than that those observed in pig liver DNA. On the other hand, there was no effect of the isolation method on the level of 8-OH-dA in pig liver DNA. It is interesting to note that the level of (5′*S*)-cdA was more than 3-fold greater than that of 8-OH-dA in pig liver DNA and in ct-DNA.

## DISCUSSION

Most oxidatively induced DNA lesions can be released from DNA as free bases by acidic hydrolysis. In contrast, the presence of the 8,5′-covalent bond in 8,5′-cyclopurine-2′-nucleosides renders these lesions unsuitable to be released by acidic hydrolysis. Understanding the appropriate enzymic hydrolysis conditions for the quantitative release of free 8,5′-cyclopurine-2′-nucleosides from DNA is important for the accurate measurement of the levels of these lesions in DNA samples, and for comparison with the levels of other oxidatively induced DNA lesions. The earliest studies on the measurement of 8,5′-cyclopurine-2′-nucleosides in DNA samples utilized a cocktail of enzymes including DNase I, snake venom phosphodiesterase, spleen phosphodiesterase and alkaline phosphatase to release these compounds, which were subsequently detected by GC/MS (8,9,15). More recently, similar enzymic methods plus the use of nuclease P1 have been applied to release 8,5′-cyclopurine-2′-nucleosides from DNA for detection by LC/MS (16–18). In contrast to these findings, a previous publication had claimed that free (5′*S*)-cdA could not be released using a similar combination of enzymes from two oligodeoxynucleotides, a 14mer and a 22mer containing this lesion with the sequence of d(GcAC) and d(GcAT), respectively (19). Nuclease P1 with bacterial phosphatase only released a trimer with the sequence d(GcAC) in addition to normal deoxynucleosides from the 14mer after 24 h of hydrolysis. Further treatment of the trimer did not result in the release of dG, dC or (5′*S*)-cdA. Snake venom and calf spleen phosphodiesterases did not hydrolyze the 14mer, either, and failed to cleave the bond between (5′*S*)-cdA and dG or dC.

Because our earlier studies used genomic DNA samples as opposed to oligonucleotides, in the present work, we reinvestigated the ability of different enzymes to release free (5′*S*)-cdA from four different dinucleotides and eight oligodeoxynucleotides containing this lesion, and also from genomic DNA samples. This is important because a complete release of this lesion from DNA is essential for accurate measurement in biological samples to determine its biological effects in certain diseases. One of our oligodeoxynucleotides contained the same sequence d(GcAT) as the 22mer in the previous work (19). Our results should therefore be directly comparable with those of the previous work. In contrast to the findings described above, we clearly show that free (5′*S*)-cdA can be released from dinucleotides, oligodeoxynucleotides and DNA by enzymic hydrolysis. Indeed, three oligodeoxynucleotides containing the sequence d(GcA) (4–6, Table 1) and the dinucleotide d(GcA)-3′-p were most easily hydrolyzable to release (5′*S*)-cdA among the oligodeoxynucleotides and dinucleotides tested. Even 6 h of hydrolysis released >85% of (5′*S*)-cdA from this sequence context, and the hydrolysis was complete after 24 h. The key phosphodiesterase appears to be snake venom phosphodiesterase, since we found that omission of spleen phosphodiesterase had no effect on the release of (5′*S*)-cdA from DNA. These results are also consistent with our previously published work on this lesion (15–18). We have no explanation as to why the previous authors failed to observe the release of (5′*S*)-cdA.

The differing ability of the two phosphodiesterases to release (5′*S*)-cdA may be understandable based on their mechanisms of action. Snake venom phosphodiesterase hydrolyzes in the 3′→5′ direction along the DNA backbone, releasing 2′-deoxynucleoside-5′-monophosphates, whereas spleen phosphodiesterase proceeds in the 5′→3′ direction, leading to 2′-deoxynucleoside-3′-monophosphates. Therefore, when hydrolyzing DNA containing (5′*S*)-cdA, spleen phosphodiesterase would have to cleave the highly distorted bond between the phosphate and the 5′-carbon of the lesion. In contrast, snake venom phosphodiesterase would cleave between the phosphate and the 3′-carbon of the normal nucleotide 5′ to the lesion, a bond that is likely to be less distorted.

We also observed significant effects of the 5′-neighboring sequences on the release of (5′*S*)-cdA. A dT residue 5′ to the (5′*S*)-cdA had the strongest inhibitory effect on the release of the latter, followed by dC, dA and dG. Even after 48 h of hydrolysis, the dinucleotide containing a 5′-dT to the lesion could not be completely cleaved. On the other hand, oligodeoxynucleotides containing the same sequence were mostly hydrolyzed after 24 h of hydrolysis with the complete hydrolysis after 48 h. Thus, it is important to consider the differences in the absolute amounts of the lesion used here and the levels likely to be found in the actual biological DNA samples. If (5′*S*)-cdA is present in DNA at a level of less than one lesion per $10^6$ normal DNA nucleotides, DNA would contain this lesion diluted in a vast excess of normal deoxynucleotides. Therefore, for most DNA samples containing baseline or slightly elevated levels of this lesion, such as after 2 Gy of ionizing radiation, 24 h of hydrolysis should be sufficient for its accurate measurement. Indeed, our results showed that (5′*S*)-cdA was completely released from genomic DNA samples, ct-DNA, and ct-DNA gamma irradiated at 2 Gy even after 6 h of hydrolysis, as evidenced from the absence of increase in the yield of this compound after 6 h, and the absence of dinucleoside monophosphates containing this lesion at the 3′-position, especially the most hydrolysis-resistant d(TcA).

Another paper also reported the limited release of (5′*R*)-cdA, (5′*R*)-cdG and (5′*S*)-cdG from oligodeoxynucleotides by nuclease P1 and alkaline phosphatase, and no release by a mixture of DNase I, and snake venom and spleen phosphodiesterases (26). This is in contrast to our previous work on the facile release of these compounds by a similar enzyme combination from DNA, even after gamma irradiation of DNA as detected by GC/MS (8,9,15). More recently, we confirmed these findings using similar enzymic methods and detection

by LC/MS (16–18). Since we did not have any dinucleotides or oligodeoxynucleotides containing (5′*R*)-cdA, (5′*R*)-cdG or (5′*S*)-cdG, we could not test the release of these compounds under our experimental conditions using LC/MS and GC/MS. In the present work, however, we did observe the release of these compounds from DNA samples (data not shown), confirming our previous work (8,9,15–18).

Recently, a $^{32}$P-postlabeling assay and co-chromatography were used to show that a subset of endogenous lesions (Type II I-compounds) released from DNA by a different type of enzymic hydrolysis are dinucleotides containing each of the normal deoxynucleotides 5′ to (5′*S*)-cdA (20). These results indicated that the phosphodiester bond 5′ to the lesion resists cleavage by the enzymes used in the postlabeling assay, i.e. micrococcal nuclease, spleen phosphodiesterase and nuclease P1. To confirm this observation, we hydrolyzed dinucleotides and oligodeoxynucleotides containing (5′*S*)-cdA with these three enzymes under those reaction conditions, and monitored the release of this lesion using LC/MS. We found that 1–19% of the (5′*S*)-cdA under these conditions was released as free cdA from dinucleotides and oligodeoxynucleotides, consistent with the previous observations (20). On the other hand, these authors also calculated the levels of (5′*S*)-cdA lesions in samples from rat tissues based on the resistance to hydrolysis of the phosphodiester bond 5′ to the lesion. Our current observations showing that some amount of free (5′*S*)-cdA can be released from DNA under the enzymic conditions used in the postlabeling assay, raises the possibility that the levels of cdA reported in that work may have represented an underestimation of the true values.

Since LC/MS is suitable for measuring both 8,5′-cyclopurine-2′-nucleosides and other oxidatively induced DNA lesions that are repaired by BER, we could directly compare the amounts of (5′*S*)-cdA, 8-OH-dG and 8-OH-dA in the same DNA samples. In pig liver DNA, the levels of (5′*S*)-cdA were ∼4−7-fold lower than the levels of 8-OH-dG, but were ∼3-fold higher than the levels of 8-OH-dA. In commercial preparations of ct-DNA, the levels of 8-OH-dG and 8-OH-dA were ∼20- and 10-fold, respectively, higher than those in pig liver DNA. In contrast, the levels of (5′*S*)-cdA were essentially the same. These observations suggest that the ct-DNA became oxidized during preparation and/or storage to form 8-OH-dG and 8-OH-dA, but that this oxidation did not increase the level of (5′S)-cdA. The lack of artifactual formation of (5′*S*)-cdA is consistent with other data showing that molecular oxygen inhibits the formation of 8,5′-cyclopurine-2′-deoxynucleosides because of its diffusion-controlled reaction with the sugar radical prior to cyclization. Finally, as a methodological point, we found that while the addition of desferroximine to the lysis buffer used in the phenol–chloroform method (21) significantly reduced the levels of 8-OH-dG and 8-OH-dA, the lowest levels of these lesions were found in samples that were prepared using a high-salt based method (22,23), which is relatively simple to perform and avoids the use of phenol.

In conclusion, our results clearly show that (5′*S*)-cdA is readily released from dinucleotides, oligodeoxynucleotides and DNA containing this lesion as evidenced by the use of LC/MS. We do not know why the previous authors (19) failed to observe the release of (5′*S*)-cdA. The experimental conditions used in the present study permits the complete release of (5′*S*)-cdA as a free nucleoside and can be used for the accurate measurement of this lesion in biological DNA samples at levels less than one lesion per $10^6$ DNA nucleotides. Using this methodology, we find that (5′*S*)-cdA is present in samples at levels that are within an order of magnitude of those of 8-OH-dG, and higher than 8-OH-dA. When considered along with the powerful effects of 8,5′-cyclopurine-2′-nucleosides on both replicative (11), and bypass (13) DNA polymerases, RNA polymerase II (10), exonucleases (13) and transcription factor binding (14), these results serve to emphasize further the biological significance of this unique form of oxidative DNA damage.

## ACKNOWLEDGEMENTS

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification neither imply recommendation or endorsement by the National Institute of Standards and Technology nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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