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Advances in Brief

Detection of 1,N^2-Propanodeoxyguanosine Adducts as Potential Endogenous DNA Lesions in Rodent and Human Tissues[1]

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Abstract

Our previous study (R. G. Nath and F-L. Chung; Proc. Natl. Acad. Sci. USA, *91:* 7491–7495, 1994), using a ^{32}P postlabeling method combined with high-performance liquid chromatography specifically developed for exocyclic adducts, has shown that acrolein- and crotonaldehyde-derived 1,N^2-propanodeoxyguanosine adducts (AdG and CdG, respectively) are present in the liver DNA from humans and rodents without carcinogen treatment. Those findings raised important questions regarding their role as potential endogenous DNA lesions in carcinogenesis. In this study, using a similar assay, we examined a variety of tissues from untreated rats and mice (lung, kidney, brain, breast, prostate, colon, skin, and leukocytes) and detected AdG and CdG in the DNA of these tissues. More significantly, we also obtained evidence for the presence of these adducts in the DNA of human leukocytes and mammary glands. The identities of these adducts were verified by comigration of 3′,5′-bisphosphates of the ^{32}P-labeled adduct from DNA with the synthetic standards in a reversed-phase high-performance liquid chromatography. Additional proof of identities was provided by enzymatic conversion of AdG and CdG 3′,5′-bisphosphates to the corresponding 5′-monophosphates, followed by comigration with their synthetic standards. The estimated ranges of total AdG and CdG modifications in DNA of various tissues were from 0.10 to 1.60 μmol/mol guanine for rodents and 0.01 to 0.78 μmol/mol guanine for humans, based on the recoveries of external standards. This study demonstrated the ubiquity of these adducts in various tissues, suggesting their potential role as endogeneous DNA lesions in rodents and humans.

Introduction

It has become increasingly apparent that, in addition to exposure to a variety of exogenous genotoxic chemicals, cells are exposed to DNA-reactive substances of endogenous origin. Oxygen free radicals, malondialdehyde, and α,β-unsaturated aldehydes (enals) are among the endogenous substances capable of causing DNA damage (1, 2). Like malondialdehyde, enals are a group of highly toxic and DNA reactive substances identified primarily as products of lipid peroxidation (3). Lipid peroxidation has been implicated in carcinogenesis, although the underlying mechanism has yet to be elucidated (4). Lipid peroxidation-derived enals such as acrolein, crotonaldehyde, *trans*-4-hydroxy-2-nonenal, and malondialdehyde can modify DNA bases with the formation of exocyclic adducts (5–8). Alternatively, enals can be readily epoxidized, and their epoxides yield DNA bases with etheno modifications (6, 8). In addition to the endogenous formation, acrolein and crotonaldehyde also occur in the environment as components of tobacco smoke and automobile exhaust, and are produced upon cooking fat-containing foods (9–11). Traces of these aldehydes are also found in several foods including fruits, vegetables, red wine, and other alcoholic beverages (12). AdG[3] and CdG are detected in animals treated with carcinogens such as cyclophosphamide and NPYR (13,14). Immunoassays have shown that 1,N^2-propanodeoxyguanosine adducts are present in the DNA of *Salmonella typhimurium* tester strains and cultured Chinese hamster ovary cells treated with acrolein and crotonaldehyde (15, 16). Site-specific mutagenesis studies using a structural analogue have demonstrated that the propano adduct causes base substitutions, and frame-shift (deletion) mutation in bacterial and mammalian host systems (17, 18).

We have recently developed a ^{32}P postlabeling method specifically for the detection of AdG and CdG (19). The method combines HPLC with the ^{32}P postlabeling technique, allowing detection of the stereoisomers of AdG and CdG (Fig. 1). With this method, we have detected AdG and CdG in the liver DNA obtained from humans and untreated rodents. These results raised questions about (*a*) whether these adducts are prevalent background DNA lesions in tissues, (*b*) what are the pathways or sources for their formation, and (*c*) what is their potential role in carcinogenesis. The main purpose of this study is to determine whether AdG and CdG are prevalent DNA adducts in various human and rodent tissues.

Materials and Methods

Materials. Nuclease P1, micrococcal nuclease, RNase A and T1, protease, and triethylamine were purchased from Sigma Chemical Co. (St. Louis, MO). Spleen phosphodiesterase was obtained from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP was obtained from Amersham (Arlington Heights, IL). Polyethyleneimine TLC sheets were purchased from Machery Nagel (Duren, Germany). AdG and CdG 5′-monophosphates and 3′,5′-bisphosphates were prepared by a previously described method (5, 19).

Human Subjects. Ten ml of blood were drawn from two male (a 26-year-old smoker and a 42-year-old nonsmoker) and two female (a 30-year-old nonsmoker and a 55-year-old smoker) healthy volunteers. To separate leukocytes, the samples were incubated with 20 ml NaCl (50 mM) for 15 min at 4°C and centrifuged at 4000 × *g* for 10 min. The leukocyte pellet was transferred to another tube, and the same treatment was repeated twice. DNA was isolated by the method described below. Human mammary DNA, isolated using Gupta's method (20) from three breast reduction surgery samples (ages 17–32 years), was generously provided by Dr. Donghui Li (M. D. Anderson Cancer Center, Houston, TX).

Animals. Female A/J mice (25–30 g, 4 months old) and male and female F-344 rats (200–300 g, 3–6 months old) were purchased from Charles River Laboratories (Kingston, NY) and housed in the animal facility (25°C, 12-h light/dark cycle) with free access to modified AIN-76A diet and tap water. After 2 weeks of acclimatization, all animals were sacrificed, and various tissues were removed (skin from mice; lung, kidney colonic mucosa, prostate, and leukocytes from male rats; and mammary fat pads from female rats) and frozen at −80°C until DNA isolation.

DNA Isolation. DNA was isolated (from ~1.0 g tissue) by a modified Marmur's procedure (21). For DNA from colonic mucosa (200–250 mg tissue), only a single precipitation was carried out for better yield. Whole brain

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[3] The abbreviations used are: AdG, acrolein-derived 1,N^2-propanodeoxyguanosine; CdG, crotonaldehyde-derived 1,N^2-propanodeoxyguanosine; HPLC, high-performance liquid chromatography; NPYR, *N*-nitrosopyrrolidine.

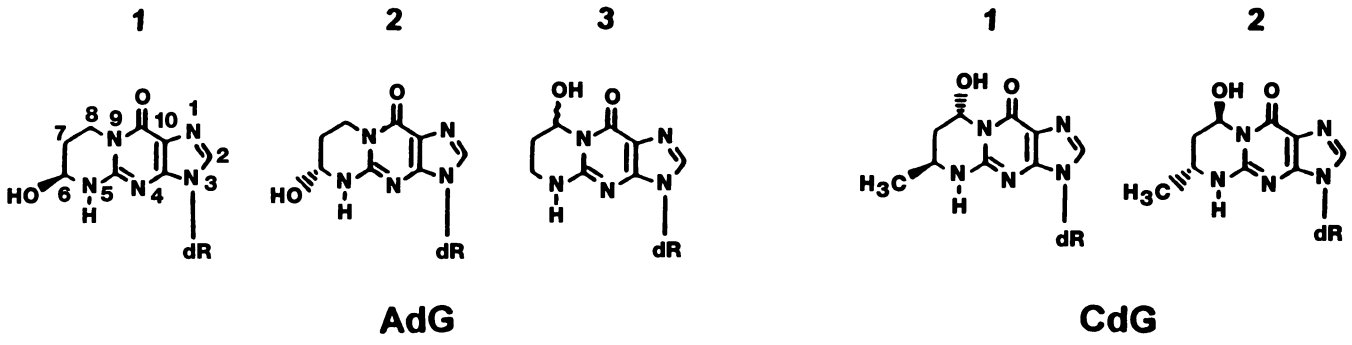

Fig. 1. Structures of AdG and CdG isomers. *dR*, deoxyribose.

DNA (from 3–4-month-old male Sprague-Dawley rats), isolated by Marmur's method, was provided by Dr. Cliff Conaway (American Health Foundation). The purity of DNA was assured by the 260:280 nm ratio (>1.8), and DNA was stored at −80°C until analysis.

Detection of Adducts by ^{32}P Postlabeling Method Combined with HPLC. The assay used for adduct detection and quantification was similar to the previously published method (22). The adduct peaks were quantified by reversed-phase HPLC-radioflow analysis using the synthetic AdG and CdG as external standards for each set of samples from a given tissue. One blank H_2O sample was assayed by the same method before each set of DNA samples to ensure that the system was free of contamination. We have made the following changes to improve the method: (*a*) the initial HPLC prepurification is carried out using a faster system with one column and 1 mM Tris-HCl as buffer A (HPLC system 1, described below). This step reduced the Tris concentration in the collected adduct fraction and, thus, improved the labeling efficiency (13); (*b*) only the fraction corresponding to AdG 2 and 3 was collected for labeling. AdG 1, the earlier eluting AdG isomer, which elutes closely with dAMP, was not collected. This step minimized residual contamination by dAMP, which not only decreases labeling efficiency but also interferes with the final analysis. The detection of AdG 1 is not affected because it is established that AdG 1 and 2 exist in equilibrium with equal amounts (5); (*c*) after labeling, adduct bisphosphates were first purified by a reversed-phase HPLC as described previously (22), followed by a simultaneous purification of AdG and CdG using an ion pair HPLC column (system 3). The purified adducts were finally analyzed by reversed-phase HPLC-radioflow system 4; (*d*) for a more accurate estimate of recoveries, AdG and CdG 3′-monophosphate standards were labeled in the presence of Tris-HCl (pH 5.8) in a concentration identical to the Tris concentration of collected adduct fractions; and (*e*) for confirming the identities of adducts, the comigrating radioactive material was collected from system 3 and then purified to remove NaH_2PO_4, which retarded the hydrolysis by nuclease P1 on a citrate-succinate reversed-phase HPLC (system 5) instead of the ion pair HPLC used previously (22).

HPLC Systems. Details of instruments and columns used are as outlined in our previous paper (22). Various HPLC systems used are as described below. The flow rate for all of the systems was 0.6 ml/min.

System 1 comprised one Burdick & Jackson (Baxter Healthcare, McGaw

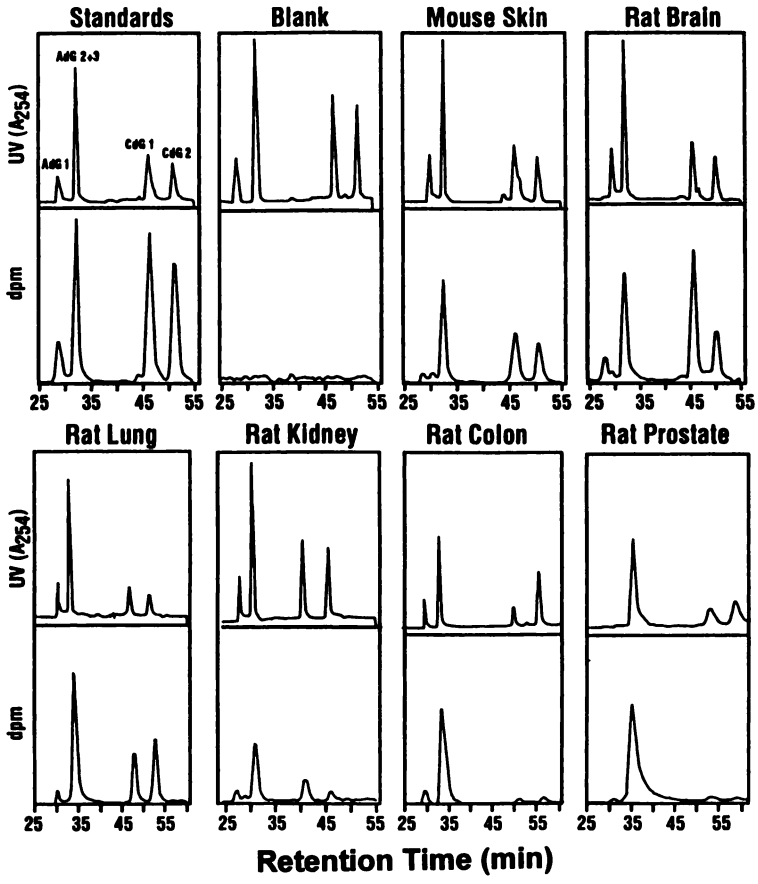

Fig. 2. HPLC chromatograms showing comigration of the purified radioactive peaks obtained from DNA of various rodent tissues with the synthetic UV standards of the 3′,5′-bisphosphates of AdG and CdG. Variation in retention times is due to column aging over several months. The dpm scale varied from 100–1500; however, sizes of the peaks should not be quantitatively compared because different volumes of DNA fractions were labeled and the analysis was done during different stages of radioactivity decay. (See Table 1 for the comparison of the range of adduct levels in various tissues.)

Park, IL) 5-μm 4.6- × 250-mm C_{18} reversed-phase column [A, 1 mM Tris-HCl (pH 5.8); B, methanol:water, 50:50; 0–30% B in 30 min].

System 2 comprised two columns identical to that used in system 1 connected in series [A, 50 mM NaH_2PO_4 (pH 5.2); B, methanol:water, 50:50, 0–25 min, 100% A, then 0–32% B, 25–65 min].

System 3 comprised one column as in system 1 [A, 25 mM triethylamine phosphate (pH 6.5); B, methanol:water, 50:50; 0–30% B in 60 min].

System 4 was similar to system 2, except that the pH of buffer A was 5.8 (0–15% B in 60 min).

System 5 comprised two columns as in system 2 [A, 10 mM sodium citrate and 15 mM sodium succinate (pH 5.8); B, methanol:water, 50:50; 0–15% B in 60 min].

Results and Discussion

Recently, we reported the detection of the exocyclic propano adducts AdG and CdG in the liver DNA of humans and rodents without carcinogen treatment (22). It was speculated that these DNA adducts may be produced by endogenous pathways such as lipid peroxidation. Considering the potential implications of these observations, in this study, we examined a variety of tissues of rodents and humans and demonstrated that AdG and CdG are indeed prevalent background DNA adducts in rodent and human tissues. The method of detection used in this study was based on a previously described ^{32}P postlabeling assay with some modifications. The ^{32}P postlabeling method is presently thought to be one of the most sensitive methods for detection of DNA adducts. However, this technique generally suffers from a number of drawbacks, most notably, poor quantification and nonspecific identification. These problems were alleviated in our studies by the use of synthetic adducts as external standards and by combining with HPLC for purification and quantification. In the present study, changes were made to the previous method to minimize the interference peaks, improve the labeling efficiency, obtain better estimates of recovery, and shorten the assay time (see "Materials and Methods"). The current method offered remarkable sensitivity, allowing the detection of as low as 0.1 fmol of AdG and CdG. Further, this method usually needs less than 50 μg of DNA sample, an important advantage for studies of human samples. However, with all of these improvements, the assay is still hampered by a lack of internal standards for more accurate quantifications.

Typical HPLC chromatograms showing comigrations of radioactive peaks obtained from DNA of various rodent tissues with the synthetic adduct standards are shown in Fig. 2. Comigrations were also demonstrated with DNA samples from human leukocytes and mammary glands. Fig. 3 shows the chromatograms obtained from these two tissues of rats and humans. Additional proof of identities of adducts in each tissue was obtained from conversion of the labeled adduct 3′,5′-bisphosphates with nuclease P1 to the corresponding 5′-monophosphates. Comigration of the resulting adduct 5′-monophosphates with the synthetic adduct 5′-monophosphates confirmed the identities of the adducts. Fig. 4 depicts the comigration of purified AdG and CdG 5′-monophosphates from rat leukocyte DNA with their UV standards after conversion. Similar results were obtained for all other tissues. Thus, the present study provides unambiguous evidence for the presence of AdG and CdG in a variety of tissues of rodents and humans. The consistent detection of these lesions in tissue DNA examined thus far suggests that it is highly likely that they are also to be found in tissues not yet examined.

Levels of AdG and CdG in tissue DNA were estimated based on the recoveries of the external standards, as shown in Table 1. The intra- and interassay variabilities were in the range of 16–35% and 8–19%, respectively, as determined by analyzing a given sample 6 times in 3 separate assays. A wide variability was observed for each tissue, possibly due to individual variations. It is noted, however, that in all

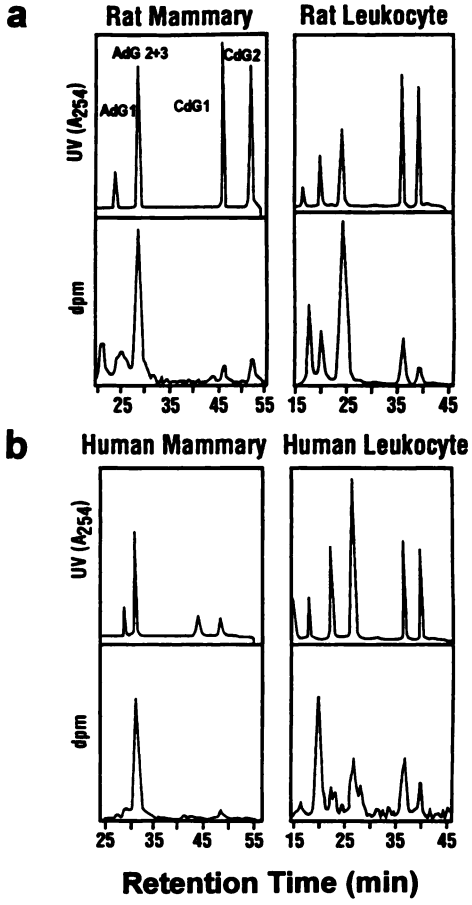

Fig. 3. HPLC chromatograms showing comigration of the purified radioactive peaks obtained from leukocyte and mammary gland DNA of rats (*a*) and humans (*b*) with AdG and CdG standards. For human leukocyte DNA, the early peak eluting at 20 min was identified as deoxyadenosine 3′,5′-bisphosphate. This chromatogram obtained from human leukocytes, unlike others, was obtained by the method described earlier (22).

tissues the AdG levels appeared to be higher than CdG. Among AdG isomers, AdG 3 was the major adduct detected, and the levels of AdG 1 were too low to be quantified or detected in most tissues. Whether this is due to its stereoselective formation and/or poor repair is presently not known. It also appeared that each tissue had a distinct pattern of adduct distribution. For example, brain seemed to contain higher CdG than most other tissues, whereas prostate and mammary glands contained primarily AdG. The tissue-specific pattern and stereoselectivity of adduct detection suggest that these adducts are not products of artifacts from DNA isolation. In addition, we observed comparable levels of AdG and CdG in rat liver DNA isolated with and without the addition of antioxidant vitamins (vitamins C and E) plus sodium mercaptoethane sulfate (an enal scavenger) during DNA isolation. The amount of AdG and CdG detected in DNA could represent the basal levels of these adducts in each tissue, possibly a steady state resulting from continuous formation and repair. It is, however, not yet clear whether an endogenous or exogenous route of exposure is predominantly responsible for these DNA modifications. Lipid peroxidation is an endogenous source of acrolein and crotonaldehyde (3). Acrolein is also a product of polyamine oxidation (23). Crotonaldehyde has been detected in human blood (24). A major source of human exposure to acrolein is cigarette smoke (10); however, the levels of AdG in leukocyte DNA were not significantly different between smokers and nonsmokers in the small number of samples that we examined in this study. CdG was detected in the liver DNA of the NPYR-treated rats (13). Some of the tissue CdG could be attributed to

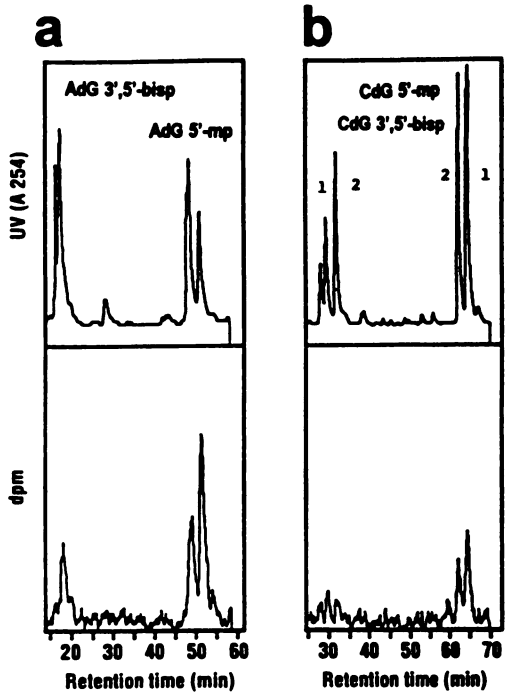

Fig. 4. Confirmation of AdG and CdG in rat leukocyte DNA after conversion of the purified labeled adduct 3',5'-bisphosphates to 5'-monophosphates with nuclease P1 (see "Materials and Methods"). *Upper panels*, synthetic UV standards; *lower panels*, radioactive adduct peaks obtained from leukocyte DNA. After conversion of the 3',5'-bisphosphates, a major radioactive peak was found to comigrate with the 5'-monophosphates of AdG 2 and 3 in *a*. A residual radioactivity was still detected for the 3',5'-bisphosphate of AdG 2 and 3 due to incomplete conversion. In *b*, the conversion of the 3',5'-bisphosphates of CdG resulted in a reversed eluting sequence for CdG 1 and 2 at the 5'-monophosphate level. After conversion, CdG 1, the predominant CdG isomer (see Fig. 3) and CdG 2 comigrated with the corresponding 5'-monophosphates of CdG 1 and 2.

Table 1 *Range of AdG and CdG adducts (μmol/mol guanine) in various rodent and human tissues*

Tissue	AdG3	CdG1	CdG2	AdG + CdG
Mouse skin (5)[a]	0.270–0.557	0.021–0.076	0.020–0.073	0.311–0.706
Rat brain (3)[b]	0.267–0.431	0.213–0.420	0.184–0.271	0.664–1.122
Rat lung (4)	0.371–0.647	0.048–0.166	0.069–0.303	0.488–1.116
Rat kidney (4)	0.055–0.165	0.042–0.105	0.014–0.107	0.111–0.377
Rat colon (5)	0.245–0.934	0.010–0.068	0.034–0.067	0.289–1.069
Rat prostate (6)	0.088–0.673	0.007–0.047	0.011–0.078	0.106–0.798
Rat mammary (4)	0.106–0.134	0.004–0.013	0.005–0.010	0.115–0.157
Rat leukocyte (3)	0.154–0.290	0.058–0.094	0.016–0.027	0.228–0.411
Human mammary (3)	0.010–0.660	0.004–0.077	0.006–0.051	0.020–0.788
Human leukocyte (3)[c]	0.003–0.025	0.005–0.025	0.003–0.011	0.011–0.061

[a] Number in parenthesis is the number of samples analyzed.
[b] Sprague-Dawley rat.
[c] DNA of this tissue was assayed by the previous method (22).

exposure to NPYR, a rat liver carcinogen found in the environment and in processed meats (25). NPYR is also a product of endogenous nitrosation of pyrrolidine, which is a widely occurring secondary amine (26). Judging from the high reactivity of acrolein and crotonaldehyde, particularly toward cellular sulfhydryls, it is conceivable that only a very small fraction of exogenously exposed enals may reach DNA. To maintain the levels of modifications detected in this study, it would appear that cells have to be constantly exposed to amounts of enals considerably higher than what is known to be present in the environment. Alternatively, the levels of adducts detected in rodents could be a result of lifetime accumulation due to inefficient repair. In this context, it is plausible that DNA from younger subjects will contain lower levels of adduct than older subjects. The age-related effect on the levels of AdG and CdG is currently being investigated.

The detection in various tissues of significant levels of exocyclic DNA modifications suggests their endogenous origin and poses important questions about their potential roles in carcinogenesis. Thus far, the strongest evidence for their roles in carcinogenesis, perhaps, comes from studies that showed increased levels of these adducts in the liver DNA of rodents treated with NPYR (13), and from studies of site-specific mutagenesis in *E. coli* and mammalian cells that showed that 1,N^2-propanoguanine is a mutagenic lesion, inducing primarily G to T transversions (18). Besides the exocyclic adducts described here, many other endogenous DNA lesions have been identified in tissues, including oxidative damage, deamination, depurination, and alkylated bases (1). Exocyclic etheno and the malondialdehyde-derived adducts have been detected recently in humans and rodents (27, 28). Thus, exocyclic adducts appear to have emerged as a novel class of endogenous DNA damage. Future studies should focus on the identification of endogenous sources for the formation of these adducts and their role in carcinogenesis.

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