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Comparison of bisulfite modification of 5-methyldeoxycytidine and deoxycytidine residues

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ABSTRACT

Sodium bisulfite is a mutagen which can specifically deaminate more than 96% of the cytosine residues in single-stranded DNA via formation of a 5,6-dihydrocytosine-6-sulfonate intermediate. Under the same reaction conditions, only 2-3% of the 5-methylcytosine (m^5Cyt) residues in single-stranded XP-12 DNA, which has 34 mole% m^5Cyt, was converted to thymine (Thy) residues. In contrast, at the deoxynucleoside and free base levels, the same treatment with bisulfite and then alkali converted 51% and > 95%, respectively, of the m^5Cyt to the corresponding Thy derivatives. However, the rate of reaction of m^5Cyt and its deoxyribonucleoside was much slower than that of the analogous quantitative conversion of cytosine or deoxycytidine to uracil or deoxyuridine, respectively. The much lower reactivity of m^5Cyt and its derivatives compared to that of the unmethylated analogs is primarily due to a decrease in the rate of formation of the sulfonate adduct.

INTRODUCTION

Sodium bisulfite causes the specific deamination of cytosine (Cyt) residues of single-stranded DNA through formation of a 5,6-dihydrocytosine-6-sulfonate intermediate (1,2). This cytosine-bisulfite adduct undergoes deamination at acid pH in the presence of bisulfite (3) and quantitatively eliminates bisulfite at alkaline pH. The mutagenic action of sodium bisulfite on bacteriophage (4,5), bacteria (6), animal viruses (7), yeast (8), and higher plants (9) has been demonstrated and, in all examined cases, has been shown to involve specifically G:C → A:T transitions (5-7).

The reaction of 5-methylcytosine (m^5Cyt) with sodium bisulfite has been reported to yield thymine (Thy) with a 5-methylcytosine-bisulfite adduct as an intermediate (10-12). 5-Methylcytosine residues are found as a minor component in the DNA of many procaryotes and most studied eucaryotes (13,14). A wide variety of organisms have a specific repair system to eliminate uracil residues from DNA (15-17). Given this ubiquitous repair system and the fact that Thy is a normal component of DNA, we

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postulate that a m^5Cyt to Thy conversion in DNA is more likely to yield a mutation than the corresponding Cyt to uracil (Ura) conversion. Therefore, the reaction with bisulfite of m^5Cyt, 5-methyldeoxycytidine (m^5dCyd), and DNA containing m^5Cyt as a major base was analyzed and compared to the analogous reactions of Cyt, deoxycytidine (dCyd), and DNA containing nonmethylated Cyt residues.

MATERIALS AND METHODS

Chemicals and enzymes: Bases and nucleosides were from Sigma, except for m^5dCyd, which was from Vega Biochemicals. Nuclease P1 was from Boehringer Mannheim. Bacterial alkaline phosphatase (Type III) was purchased from Sigma.

Reaction of sodium bisulfite with bases or nucleosides: Free bases and nucleosides (17-23 mM) were reacted with 3 M sodium bisulfite, pH 5.5, 0.5 mM hydroquinone at 37°C under nitrogen. Aliquots were withdrawn at various intervals and diluted 200-fold with either 0.2 M sodium citrate buffer, pH 3.1; 0.2 M glycine-NaOH buffer, pH 9.6; or 1 M NaOH. The ultraviolet absorption was measured after at least 20 min at room temperature. To calculate the concentration and mole fraction of Cyt, m^5Cyt, dCyd, or m^5dCyd and their deaminated derivatives after reaction with bisulfite, the molar extinction coefficients for the aminated and deaminated derivatives were determined. Simultaneous equations for the absorption of appropriate mixtures of pyrimidines at their respective wavelength maxima were solved (3,20). For example, at pH 3.1 the concentrations of m^5dCyd and dThd (C_{m^5dCyd} and C_{dThd}) in an aliquot from a reaction mixture of m^5dCyd and sodium busulfite can be determined from the following equations:

$$C_{m^5dCyd} = (1.00\ A_{287} - 0.32\ A_{267}) \times 10^{-4}$$

$$C_{dThd} = (1.34\ A_{267} - 0.69\ A_{287}) \times 10^{-4}$$

Because the bisulfite adducts of pyrimidines have negligible absorption at greater than 240 nm (12), the amount of bisulfite adduct remaining can be determined from the difference between the original concentration of the m^5dCyd and the sum of the concentrations of m^5dCyd and dThd after the reaction.

DNA Preparation and DNA-bisulfite reaction: For preparing *X. oryzae* DNA exclusively radiolabeled in its Cyt residues, a thymine auxotroph of *X. oryzae* was grown in XMS medium (19) supplemented with dThd and [2-^{14}C]uracil (21). To label bacteriophage XP-12 DNA to equal specific activity in Thy and m^5Cyt residues, the phage were propagated on *X. oryzae* in the presence of [2-^{14}C]uracil as previously described (21). Unlabeled

DNA was isolated by standard methods (22). DNA at a concentration of 50-100 μg/ml was reacted with 3 M sodium bisulfite, pH 5.5, 0.5 mM hydroquinone at 37°C for 48 or 96 h. The bisulfite reaction was terminated by dialyzing at 0°-4°C against 5 mM sodium acetate, pH 5.2, 0.5 mM hydroquinone, and then against 0.5 mM sodium acetate, pH 5.2. Half of the dialysate was directly lyophilized and the other half was incubated with 0.1 M NH_4OH for 30 min at 37°C, and then lyophilized, rehydrated and lyophilized again.

Nuclease Pl digestion and chromatography: For thin layer chromatography (TLC) the lyophilized DNA samples were dissolved in 10 mM sodium acetate, pH 5.2 and hydrolyzed to 5'-mononucleotides by adding $ZnSO_4$ to 1 mM and nuclease Pl (80 units per mg of DNA) and then incubating at 37°C for 2 h. The hydrolysates plus internal markers were directly applied to a 250 μ cellulose, thin-layer plate (Avicel; Analtech) and chromatographed in only one dimension with isobutyric acid:H_2O:NH_4OH (66:20:1, v/v) or in a subsequent second dimension with isopropanol:1M ammonium acetate (7:4, v/v) or 95% ethanol:1 M ammonium acetate (7:3, v/v). After chromatography, the distribution of radioactivity was determined as described previously (19). For high performance liquid chromatography (HPLC) the DNA was hydrolyzed to mononucleosides and chromatographed on a reverse phase column (23). Monitoring of the HPLC peaks was done simultaneously at 254 nm and 280 nm to give maximal sensitivity for the various deoxynucleosides. In one experiment the elution profile was also observed at 220 nm so that products which might have only end absorption could be detected.

RESULTS

Reaction of pyrimidine bases and deoxynucleosides with bisulfite

Figure 1 shows that Cyt and dCyd react with sodium bisulfite at pH 5.5 as monitored by the loss of ultraviolet absorption at >240 nm. Previous studies have demonstrated that the saturated product from Cyt is a bisulfite adduct, 5,6-dihydrocytosine-6-sulfonate or, as described below, the corresponding adduct of uracil (1,24). In contrast to the relatively rapid reaction of Cyt and dCyd, the reaction of m^5Cyt and m^5dCyd with bisulfite is much slower (Figure 2). The formation of 5,6-dihydrothymine-6-sulfonate from m^5Cyt and bisulfite has been briefly described previously (25,26).

The bisulfite adducts of Cyt its ribonucleoside or nucleotide derivatives undergo deamination at acid pH to form the corresponding uracil bisulfite adducts but at a much slower rate than that for adduct formation itself

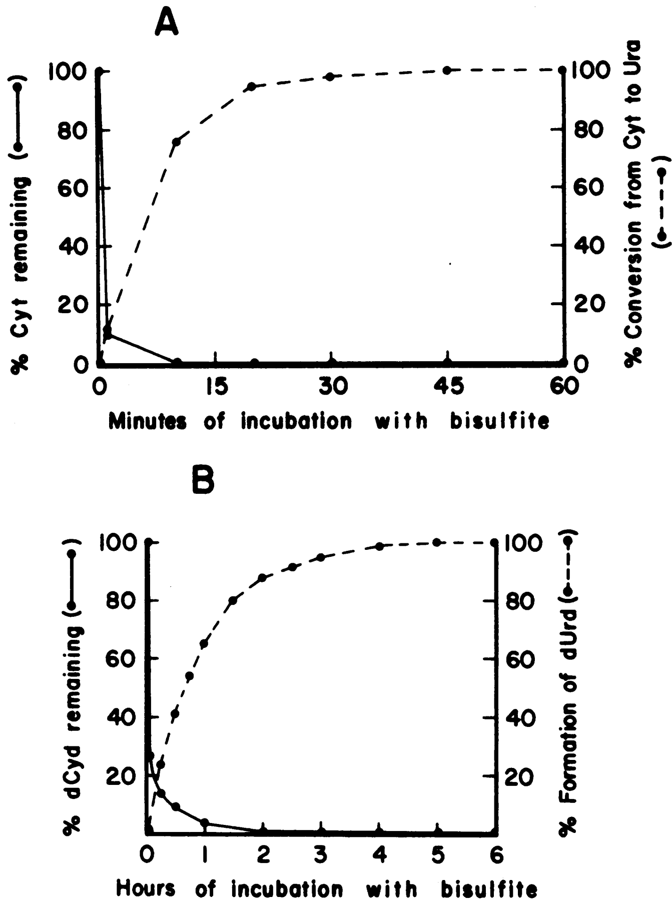

Figure 1 Incubation of cytosine (Fig. 1A) or deoxycytidine (Fig. 1B) with sodium bisulfite at pH 5.5. The sample was incubated with bisulfite and then the % Cyt or dCyd remaining was determined. Alternatively, it was subsequently treated with alkali and then the % conversion of Cyt to Ura or of dCyd to dUrd was determined.

(1,27). In alkali, elimination of bisulfite occurs rapidly regenerating the parent compounds or, if deamination has occurred at acid pH, the corresponding Ura derivatives (1,3). The loss of bisulfite with or without prior deamination was followed by treating the samples with bisulfite at pH 5.5 for various lengths of time, and then monitoring at several wavelengths the ultraviolet absorption of aliquots of the mixtures in a buffer

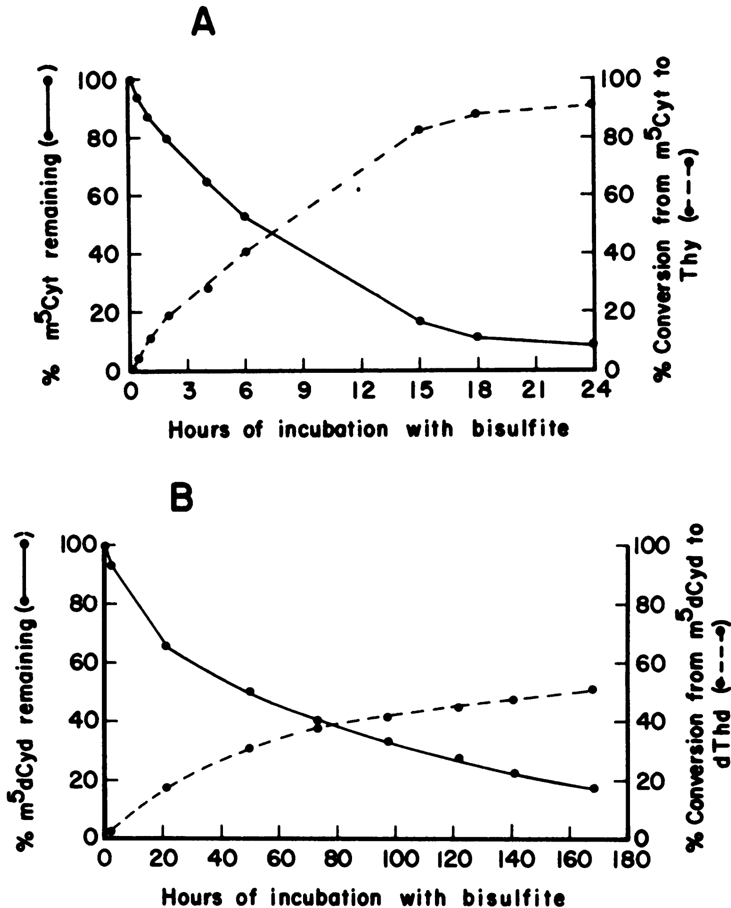

Figure 2 Incubation of 5-methylcytosine (Fig. 2A) or 5-methyldeoxycytidine (Fig. 2B) with sodium bisulfite at pH 5.5. The sample was incubated with bisulfite and then the % m^5Cyt or m^5dCyd remaining was determined. Alternatively, it was subsequently treated with alkali and then the % conversion of m^5Cyt to Thy or of m^5dCyd to dThd was determined.

of pH 3.1 or 9.6 described in Material and Methods. Figure 1 shows that deamination of both compounds was quantitative although it was much slower than the almost instantaneous formation of bisulfite adduct.

The deamination of m^5Cyt and m^5dCyd occurred much more slowly than that of their nonmethylated analogs and was difficult to drive to completion (Figure 2). Nonetheless, more than 90% conversion of m^5Cyt to Thy was

obtained after incubation with 3 M sodium bisulfite for 1 day at 37°C followed by treatment with strong alkali, namely, 1 M NaOH for 30 min at 24°C. Under analogous reaction conditions after 7 days of incubation with bisulfite (Figure 2) m^5dCyd yielded 17% m^5dCyd, 51% dThd, and 32% compounds which did not absorb ultraviolet light at >255 nm, and so, presumably, were bisulfite adducts. A portion of the bisulfite adduct population derived from m^5Cyt and m^5dCyd was resistant to elimination of bisulfite in alkali; this adduct from m^5dCyd was even more resistant than that from m^5Cyt (Figure 3). In contrast, bisulfite adducts from both dCyd and Cyt rapidly eliminate bisulfite at pH 9.6 (data not shown).

As noted by Hayatsu and coworkers (26), even at acid pH a significant portion of the bisulfite adduct of m^5Cyt eliminates bisulfite. In this study, as detected by changes in the ultraviolet absorption spectrum, 18% of m^5Cyt and 24% of m^5dCyd were converted to Thy and dThd, respectively, upon treatment with 3 M sodium bisulfite at pH 5.5 and 37°C for 96 hours. The conversion of m^5dCyd to dThd was also confirmed by HPLC.

In order to observe the effect of 5-methylation of another pyrimidine upon its reaction with bisulfite, the reactivity of dUrd and of dThd were compared. After 15 min or 1 h at 37°C in 3 M sodium bisulfite, pH 5.5, 0.5 mM hydroquinone, 72% and >99%, respectively, of the dUrd was converted to

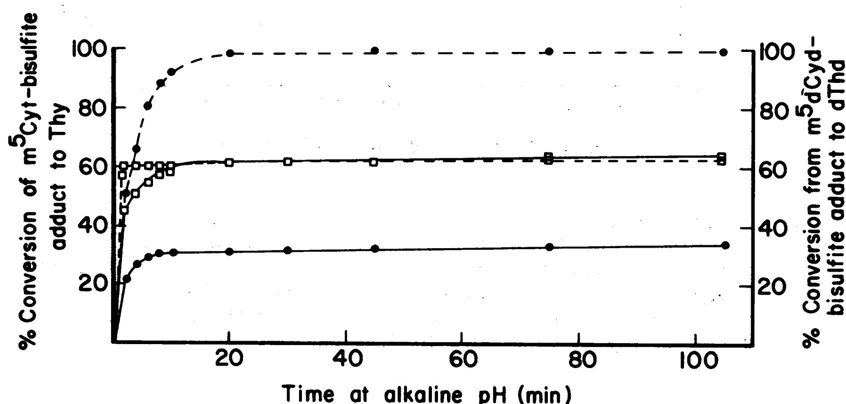

Figure 3 The stability of the bisulfite adduct of 5-methylcytosine or of 5-methyldeoxycytidine in alkali. The adducts were formed by incubation with 3 M sodium bisulfite at 37°C for 96 h for m^5Cyt and for 168 h for m^5dCyd.
●—●, m^5Cyt-bisulfite at pH 9.6
●- -●, m^5Cyt-bisulfite in 1 M NaOH
□—□, m^5dCyd-bisulfite at pH 9.6
□- -□, m^5dCyd-bisulfite in 1 M NaOH

the bisulfite adduct as detected by the loss of absorption of ultraviolet light. After 1 h and 22 h under the same conditions only 15% and 27%, respectively, of dThd was present as the bisulfite adduct. For dUrd the reaction rate increased and quantitative conversion to the adduct was still observed when 60°C instead 37°C was used for the incubation. Although the initial reaction rate increased for formation of the bisulfite adduct of dThd at 60°C compared to at 37°C, the final extent of reaction was less (15% after 22 h at 60°C) due to a partial reversal of the reaction between 6 and 22 h. For the adducts of both dThd and dUrd, incubation at pH 9.6 for 30 min at room temperature quantitatively regenerated the starting material.

Reaction of cytosine residues in DNA with bisulfite

As a GC-rich DNA, the DNA from the bacterium *Xanthomonas oryzae* (64 mole % GC and only 0.09 mole % m^5Cyt; 18,23) was chosen for reaction with sodium bisulfite. *X. oryzae* DNA specifically radiolabeled in its Cyt residues (21) was denatured, reacted with 3 M sodium bisulfite for 96 h at 37°C, hydrolyzed to 5'-mononucleotides with nuclease P1 and chromatographed on TLC plates at pH 3.8. The adduct was maintained at acid pH, at which it is maximally stable (25). As shown in Table I, 95% of the Cyt residues were converted to low R_f material. That almost all of this slow moving radioactivity is the bisulfite adduct from cytosine residues is indicated by the near equivalence of radioactivity in the low R_f region when the DNA was not subsequently treated with alkali and the amount of radioactivity in the dUMP spot when the sample was treated with alkali (Table I). In an analogous experiment with bisulfite-treated, unlabeled *X. oryzae* DNA, the R_f 0.0 - 0.1 material derived from a nuclease P1 digest was treated with alkali and then redigested with nuclease P1. Any unreacted DNA residues appearing in the low R_f region due to their inclusion in P1-resistant oligonucleotides containing the bisulfite adduct of cytosine should have been converted to mononucleotides during the second digestion after the alkali-induced elimination of bisulfite. These mononucleotides were quantitated by determining their ultraviolet absorption after elution. The results of this experiment indicated that without alkaline treatment, only approximately 20% each of the Thy and Ade residues chromatographed slowly due to their presence in P1-resistant oligonucleotides containing the cytosine- (or uracil-) bisulfite adduct even though approximately 31 mole % of the residues (>95% of the Cyt) had formed the bisulfite adduct.

In 10 mM sodium phosphate, 0.1 M NaCl (pH 7.4) at 37°C the half time for elimination of bisulfite from the cytosine-derived adduct in denatured DNA

Table I. Chromatographic analysis of enzymatic digests of denatured X. oryzae [^{14}C-Cyt]DNA treated with sodium bisulfite or sodium chloride.

Analysis		Alkaline treatment	3 M NaCl	3 M $NaHSO_3$
TLC	% of total radioactivity with R_f of 0.00-0.10	-	0.25	95.1[1] ± 0.9(2)
		+	0.55	0.97 ± 0.02(2)
	Molar ratio: dUMP/dCMP[2]	-	0.0010	1.3 ± 0.6(2)
		+	0.0012	55.8 ± 4.2(2)
	% Conversion of Cyt residues to Ura residues	-	<0.13	2.5 ± 1.0
		+	<0.13	98.2 ± 0.1
HPLC	Molar ratio: dUrd/dCyd	+	<0.0005	24.6
	% Conversion of Cyt residues to Ura residues	+	<0.07	96.1

[1]The average ± the range is given where more than one sample was reacted and analyzed. The number of samples is indicated in parenthesis.
[2]No significant amount of radioactivity was found in any region of the TLC plate other than the R_f 0.0-0.10, the dUMP and dCMP spots.

was approximately 30 h whereas for the adduct of free dCyd, it was approximately 2 h. Elimination of bisulfite gave Ura rather than Cyt residues because the adduct quantitatively underwent deamination during the prolonged incubation with bisulfite at acid pH. Thirty min of incubation with 0.1 M NH_4OH at 37°C after treatment with bisulfite at pH 5.5 for 48 h converted almost all of the Cyt residues of denatured [^{14}C-Cyt]DNA to Ura residues (Table I). This result was confirmed by HPLC analysis of digests of unlabeled X. oryzae DNA (Table I). The HPLC data also demonstrated that there was no detectable change (<2%) in the other residues of the denatured X. oryzae DNA as a result of treatment with bisulfite and then with alkali as described above.

The reaction of 5-methylcytosine residues in DNA with bisulfite

Bacteriophage XP-12 DNA was used as the source of m^{5}Cyt residues because >97% of its Cyt residues are 5-methylated (18,19). Phage XP-12 DNA labeled

to equal specific activity in all its pyrimidine residues has 69.2% of its radioactivity in m^5Cyt residues, 30.8% in Thy residues and <0.3% in Cyt residues. Phage XP-12 [^{14}C-Pyr]DNA was denatured and then reacted with 3 M sodium bisulfite at pH 5.5 for 96 h at 37°C. Pyrimidine-labeled DNA rather than specifically m^5Cyt-labeled XP-12 DNA was used because no method is currently available for specifically labeling the m^5Cyt residues of XP-12 DNA (21). After bisulfite treatment, the DNA was hydrolyzed with nuclease P1 and chromatographed under the same acid conditions used for X. oryzae DNA. Upon TLC, approximately 5% of the radioactivity had a low R_f and the ratio of dTMP to m^5dCMP increased slightly (Table II). Much of the increase in this ratio can be accounted for if more of the m^5Cyt residues than of the Thy residues is lost from the mononucleotide regions due to the reaction with bisulfite.

Incubation with bisulfite was routinely performed at pH 5.5 (3,26) for 96 h; incubation for 46 h gave approximately 7% less low R_f radioactivity from the pyrimidine residues of single-stranded XP-12 DNA and 1% less adduct from the Cyt residues of single-stranded X. oryzae DNA. The percentages of radioactively labeled material from XP-12 [^{14}C-Pyr]DNA,which form low R_f material after reaction at pH 5.0, 5.5, 6.0 or 6.6 followed by enzymatic digestion were 5.6, 4.9, 4.1, and 1.7, respectively. From the percentage of unreacted residues in X. oryzae DNA appearing in nuclease P1-resistant oligonucleotides (see above) and the observation that only pyrimidine residues form bisulfite adducts under the conditions used (1), it can be concluded that approximately 20-30% of the radioactivity in the low R_f region from digests of bisulfite-treated XP-12 DNA should be in unreacted pyrimidine residues. The rest of the radioactivity in this region (Table II) should be in the adduct. This calculation is based upon the labeling of all the pyrimidine residues in the XP-12 DNA and upon only a very small extent of adduct formation in this DNA.

In samples of denatured XP-12 [^{14}C-Pyr]DNA treated with bisulfite at pH 5.5 and then incubated with alkali, only 2.7% of the radioactivity remained near the origin of the TLC plate and the ratio of dTMP to m^5dCMP increased further (Table II). From control mixing experiments, it is known that traces of NH_4OH, or some contaminant thereof, and of bisulfite (eliminated from dialyzed bisulfite-treated DNA in alkali) slightly inhibited the nuclease P1 digestion and so are partially responsible for the low R_f material remaining after alkali treatment. Nonetheless, most of this low R_f radioactivity persisting after incubation with NH_4OH is apparently due to formation of an alkali-resistant bisulfite adduct in XP-12 DNA (compare Tables I and II).

Table II. Chromatographic analysis of enzymatic digests of denatured XP-12 [^{14}C-Pyr]DNA treated with sodium bisulfite or sodium chloride.

Analysis		Alkaline treatment	Native DNA		Denatured DNA	
			Untreated	3 M $NaHSO_3$	3 M NaCl	3 M $NaHSO_3$
TLC	% of total radioactivity with R_f of 0.00-0.10	-	<0.05	1.0	0.8[1] ± 0.2(3)	5.0 ± 0.4(3)
		+	-	0.9	1.4 ± 0.1(3)	2.7 ± 0.2(3)
	Molar ratio: dTMP/m^5dCMP[2]	-	0.453 ± 0.007(12)	0.461	0.472 ± 0.002(3)	0.485 ± 0.003(3)
		+	-	0.454	0.472 ± 0.001(3)	0.511 ± 0.001(3)
HPLC	Molar ratio: dThd/m^5dCyd	-	0.440 ± 0.009(8)	-	-	-
		+	0.434	-	0.450 ± 0.001(2)	0.483 ± 0.022(2)

[1]Data from more than one sample are expressed as the mean ± standard deviation except for experiments which were done only in duplicate; for those the range is given. The number of experiments is indicated in parentheses.

[2]No significant amount of radioactivity was found in any region of the TLC plate other than the R_f 0.0-0.10, the m^5dCMP and the dTMP spots.

Analysis by HPLC of the reaction of unlabeled XP-12 DNA with bisulfite after enzymatic digestion showed a similar increase in the m^5dCyd to dThd ratio (Table II). These data are most simply explained by a low yield reaction of m^5Cyt residues in denatured XP-12 DNA with bisulfite so as to form an adduct, part of which underwent deamination and then eliminated bisulfite to yield Thy residues. The net result was the conversion of approximately 2-3% of m^5Cyt residues in denatured XP-12 DNA to Thy residues. It also appears that approximately 2% of the m^5Cyt residues in denatured XP-12 DNA formed an alkali-resistant bisulfite adduct seen as material near the origin upon TLC of digests of bisulfite- and alkali-treated DNA (Table II). This inference is based on the observations that free m^5Cyt and m^5dCyd formed an alkali-resistant bisulfite adduct as well as an alkali-sensitive adduct (Figure 3), whereas Thy and dThd formed only an alkali-sensitive bisulfite adduct (11).

DISCUSSION

Sodium bisulfite is a mutagen present in the environment, which converts Cyt residues in single-stranded nucleic acid to the bisulfite adduct, cytosine-5,6-dihydro-6-sulfonate. This adduct can revert to Cyt residues or undergo deamination and then form Ura residues (1,2,24,28). Bisulfite has been shown to give reversible adduct formation with free Thy and dThd, but the equilibrium constant for formation of these adducts is much less favorable than that for formation of the corresponding cytosine or uracil adducts (29). In the present study, treatment of denatured DNA with 3 M sodium bisulfite at pH 5.5 and 37°C, for 96 h followed by exposure to alkali has been shown to result in conversion of >96% of the Cyt residues to Ura residues and <2% change in the other major residues of DNA (Table I). The conversion of Cyt residues to Ura residues takes place through a cytosine-bisulfite adduct, which can be deaminated at pH 5.5 in a rate limiting step and then can quantitatively undergo rapid elimination of bisulfite at alkaline pH (1,3; Fig. 1).

Deamination of m^5Cyt residues, which are found as a minor component of most studied DNAs (13,14), would result in Thy residues. The net effect of this reaction would be a G:C→A:T transition due to the formation of Thy from m^5Cyt. This alteration should be much more difficult for a cell to correct than a Cyt to Ura conversion (15-17,30). By studying the m^5Cyt-rich, XP-12 DNA (18,19), we have found that treatment with bisulfite followed by alkali can convert m^5Cyt residues in DNA to Thy residues but only with a very low yield. The same conditions which converted >96% of Cyt residues in single-

stranded DNA to Ura residues converted only approximately 2-3% of m^5Cyt residues to Thy residues (Tables I and II and Results). This lower extent of deamination of m^5Cyt residues is due primarily to 5-methylation of Cyt residues inhibiting the formation of the bisulfite adduct (Tables I and II; Figures 1-3).

The decrease in the rate of addition of bisulfite upon 5-methylation of pyrimidines (Figures 1 and 2) may be due to the electron donating properties of the methyl group, resulting in inhibition of nucleophilic attack at the 6-position (1). Consistent with this hypothesis is the finding that dThd reacts much more slowly with bisulfite than does dUrd (see Results), just as thymine reacts more slowly than does uracil (29). Furthermore, elevated temperature decreased the yield of the bisulfite adduct of thymine, probably by shifting the equilibrium (29), but not that of uracil. Another major factor determining the extent or rate of reaction of pyrimidines with bisulfite is whether they are present as the free base, deoxynucleoside, or deoxymononucleotide residue in single-or double-stranded DNA. In all cases that we examined we found that the relative reactivity was as follows: base> deoxynucleoside>deoxymononucleotide residue in single-stranded DNA>deoxymononucleotide residue in double-stranded DNA. The differences in reactivity are large. For example, 90% of free m^5Cyt reacted with bisulfite to give the adduct and, indirectly, Thy in 24 h of incubation with 3 M sodium bisulfite at 37°C and then treatment with alkali (Figure 2). In contrast, 96 h of similar incubation of single-stranded XP-12 DNA with bisulfite gave only approximately 4-5% of m^5Cyt residues forming adduct, and upon treatment with alkali, approximately 2-3% conversion of m^5Cyt residues to Thy residues (Table II and Results).

At the base level, m^5Cyt can form two diastereomeric forms of a Thy-bisulfite adduct, only one of which can eliminate bisulfite at moderate alkaline pH (25). This behavior is to be contrasted with that of the bisulfite adduct of Cyt or Ura, which readily quantitatively eliminates bisulfite in moderate alkali. At the base, deoxynucleoside, and single-stranded DNA level we found evidence for a relatively alkali-resistant component as well as for an alkali-sensitive component of the adduct population derived from m^5Cyt (Figure 3 and Table II), which are probably the cis- and transdiastereomers, respectively (26). The half-life of the bisulfite adduct from Cyt residues in DNA incubated under physiological conditions was approximately 2 days. The alkaline resistant portion of the bisulfite adduct population derived from m^5Cyt residues would be expected to have a much longer half-life making

it very long-lived in vivo unless it is removed by DNA repair systems.

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