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Alteration of Native DNA Transcription by the Mutagen Hydroxylamine

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SUMMARY

Treatment of T7 DNA with hydroxylamine inhibits transcription of this template by *Escherichia coli* RNA polymerase. At least one effect of hydroxylamine treatment on native T7 DNA is the introduction of single-strand scissions in most of the molecules.

Polymerase binding studies suggest that hydroxylamine-treated T7 DNA is somewhat similar to denatured DNA since more RNA polymerase is required for maximal retention on Millipore filters in contrast to untreated DNA. Moreover, crucial sites on the template may be altered by hydroxylamine because the inhibition in RNA synthesis is not overcome by addition of more RNA polymerase.

INTRODUCTION

Considerable attention has been focused recently on the mechanism of action of hydroxylamine and its molecular basis for mutagenesis (7–9, 13). At least one effect seems to be the alteration of pyrimidines, cytosine to a greater extent than uracil, and the reaction seems to affect pyrimidine bases in solution as well as those coupled by phosphodiester linkage into polynucleotides. Recent studies on the effect of hydroxylamine on polycytidylic acid templates for RNA polymerase have demonstrated marked inhibition of polyguanylic acid synthesis which is partially restored by addition of riboadenosyltriphosphate to the reaction mixture (9). The authors concluded that inhibition of polyguanylic acid synthesis was due to conversion of cytidine bases to a form which base-pairs with a purine other than guanine, *e.g.*, adenine. An alternative possibility suggested was the conversion of cytidine bases in poly C[2] to a form which did not base-pair at all, thus stopping transcription. Although other more nonspecific forms of damage to the template cannot be excluded, the report by Phillips *et al.* (9) suggests that hydroxylamine does not cause chain scission in the RNA template, poly C.

We have recently observed that RNA polymerase provides a very sensitive assay to measure subtle alterations in native DNA templates (A. E. Cato and O. W. Jones, submitted for publication). Since there is increasing evidence that RNA polymerase binds preferentially to pyrimidine-rich sequences in DNA (11, 12), it became of considerable interest to examine the effect of hydroxylamine on a native DNA template for RNA polymerase.

During these studies *in vitro*, we observed that in contrast to the effect upon poly C (8, 9, 13), neutralized but nondesalted hydroxylamine *stimulated* RNA synthesis catalyzed by *Escherichia coli* RNA polymerase and native T7 DNA. We soon learned that the stimulatory effect was due to the presence of salt in the neutralized NH_2OH. Desalted NH_2OH resulted in a marked decrease in T7 DNA transcription, a more rapid decrease than that reported with poly C. Hydroxylamine-treated T7 DNA can still bind to RNA polymerase in the absence of RNA synthesis although greater amounts of polymerase are required for complete retention of treated DNA on membrane filters. The most significant difference found with native T7 DNA is the appearance of single-strand scissions after treatment with hydroxylamine.

MATERIALS AND METHODS

RNA polymerase was purified through Fraction IV as described by Chamberlin and Berg (1). This fraction, assayed with salmon sperm DNA, has a specific activity of 4700. Unlabeled and ^{3}H-labeled bacteriophage T7 DNA was prepared as described previously (6). The A_{260}/A_{280} ratio of purified DNA was 1.94. Ribonucleoside triphosphates were purchased from Pabst Laboratories, Milwaukee, Wis. Isotopically labeled ribonucleoside triphosphates were obtained from New England Nuclear, Boston, Mass. and Schwarz BioResearch, Orangeburg, N. Y. Tritiated thymidine was obtained from New England Nuclear. Millipore filters were obtained from Millipore Filter Company, Bedford, Mass.; glass filters, type GF/C, were obtained from the Whatman Company. Sephadex was obtained from Pharmacia, Uppsala, Sweden. Hydroxylamine-HCl was obtained from Sigma Chemical Company, St. Louis, Mo. Hydroxylamine-HCl as a 1 M solution was neutralized to pH 6.9 with aqueous sodium hydroxide. Salt-free, recrystallized hydroxylamine was prepared according to the method of Hurd and Brownstein (4).

Assays. T7 DNA-RNA polymerase binding assays were performed as described previously (3). Care was taken to regulate the vacuum pressure for filtration to between 5 and

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[2] The abbreviation used is: poly C, polycytidylic acid.

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6 cm Hg. Assays for RNA synthesis were performed in a final volume of 0.25 ml containing 20 μmoles Tris-HCl, pH 7.9; 5 μmoles $MgCl_2$; 6.0 μmoles 2-mercaptoethanol; 50 mμmoles each of 3 unlabeled ribonucleoside triphosphates; 50 mμmoles labeled ribonucleoside triphosphate (specific activity and name of the labeled triphosphate are indicated in the appropriate chart legend). The amount of DNA and RNA polymerase added varied with each experiment. After incubation, the reaction was terminated by addition of 2.0 ml ice-cold 7% perchloric acid. The reaction mixtures were then filtered over Whatman GF/C glass discs and washed with 30 ml cold 1 N HCl, and then 95% ethanol. The discs were dried and counted in a Nuclear-Chicago liquid scintillation counter.

Ultracentrifugation. Sedimentation velocities were measured in a Kel-F centerpiece with a Spinco Model E ultracentrifuge equipped with a monochromator. Ultraviolet photographs were scanned with a Joyce-Loebl recording microdensitometer. The DNA solutions (approximately 20 μg/ml) were centrifuged at 44,770 rpm in 1.0 M NaCl (neutral pH) or 0.1 M NaOH and 0.9 M NaCl (alkaline pH). All sedimentation velocities were corrected to $s^0_{20,w}$. Additional corrections to infinite dilution were made according to Eigner and Doty (2). For certain experiments, especially ultracentrifugation, it became necessary to remove the hydroxylamine after incubation with DNA. For this purpose, we developed a rapid, simple method for removing hydroxylamine from DNA solutions. A total of 0.075 g Sephadex G-100 was weighed and washed twice with 0.01 M Tris-HCl, pH 7.9. The Sephadex was then poured into a small column, approximately 0.5 cm x 5.5 cm. After incubation of hydroxylamine with ^{3}H-labeled T7 DNA, the mixture (0.2 ml) was placed on the column and eluted with 0.01 M Tris, pH 7.9. Fractions of 2 drops each (approximately 0.1 ml) were collected. An aliquot of each fraction was counted directly in a scintillation counter while the remainder was subjected to a colorimetric assay for free hydroxylamine in the following manner. To 0.1 ml eluate was added 0.2 ml ethyl acetate followed by 0.5 ml absolute methanol. Each fraction was then made alkaline to litmus paper with 1 drop of absolute methanol saturated with aqueous potassium hydroxide. The samples were heated just to boiling in a water bath and then chilled in ice. After acidification to litmus paper with 0.5 N HCl, 1 drop of freshly prepared $FeCl_3$ was added. Each sample was diluted to 5 ml with H_2O and read in a Klett colorimeter with a No. 54 green filter. As shown in Chart 1, this simple procedure results in satisfactory separation of DNA from hydroxylamine. Recovery of DNA placed on the column is between 78 and 85%.

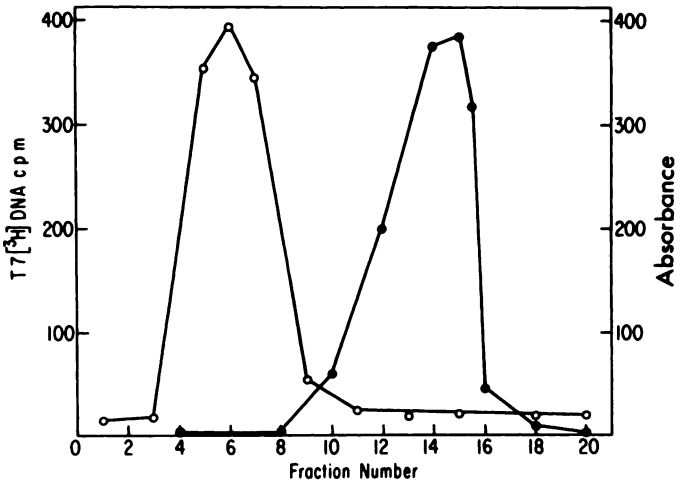

Chart 1. Separation of T7 DNA from hydroxylamine on a Sephadex G-100 column. Chromatography was performed as described in "Materials and Methods." o—o, ^{3}H-labeled T7 DNA; ●—●, free hydroxylamine as measured by colorimetric assay. A Klett colorimeter was used for the determination.

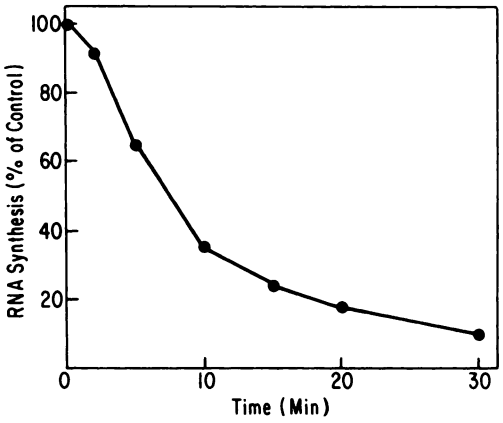

Chart 2. Inhibition of RNA synthesis from a DNA template treated with hydroxylamine. Each sample of T7 DNA was incubated at 37° in 0.5 M salt-free, recrystallized NH_2OH for the period of time shown on the *abscissa*. The NH_2OH-treated T7 DNA was then used as a template in the assay for RNA synthesis with *Escherichia coli* RNA polymerase as described in "Materials and Methods." For this experiment 50 mμmoles ATP-^{14}C, specific activity 10^6 cpm/μmole, were added to each assay. Each reaction mixture received 7 μg T7 DNA and 4 μg RNA polymerase. The assays were terminated after 10 min incubation at 37°. In this experiment untreated T7 DNA resulted in 654 cpm AMP-^{14}C incorporated into RNA.

RESULTS

Similar to earlier reports in which poly C was template (9, 13), desalted NH_2OH rapidly inhibits RNA synthesis. The experiment described in Chart 2 was performed by incubating native T7 DNA in 0.5 M NH_2OH for varying periods of time at 37° and then using the hydroxylamine-treated DNA as template for RNA synthesis. After only 5 min of incubation with NH_2OH, there is almost a 40% decrease in RNA synthesis. Synthesis rapidly declines until T7 DNA treated for 20 min with NH_2OH is less than 20% as effective as untreated T7 DNA.

The result we observe could be explained on the basis of alteration of cytidine in native T7 DNA molecules, thus making it impossible for RNA polymerase to transcribe the template. On the other hand it is conceivable that there

might be an additional effect of NH_2OH on native DNA unrelated to base-pairing. A simple way to test this possibility is by sedimentation of T7 DNA at neutral and alkaline pH after treatment with hydroxylamine.

Chart 3 demonstrates the sedimentation of *untreated* T7 DNA at neutral and alkaline pH. There is little if any evidence of single-strand scissions in this population of DNA molecules. In Chart 4, NH_2OH-treated DNA is sedimented under conditions similar to Chart 3. At neutral pH (Chart 4*a*) the DNA appears homogeneous and similar to the control experiment in Chart 3*a*. However, during sedimentation at alkaline pH, DNA molecules appear very heterogeneous and, as illustrated in Chart 4*b*, approximately one-half of the molecules are sedimenting at a rate more slowly than the $s^{50}_{20,w}$ value of 26.4 at this point. This difference is emphasized further in Chart 5 where we have plotted the *s* values obtained from the experiment shown in Chart 4. Chart 5 shows the range of sedimentation velocities throughout the entire population of DNA molecules at neutral and alkaline pH. At neutral pH, NH_2OH-treated T7 DNA has a sedimentation velocity which varies by only 4.4 units among 90% of the DNA molecules. Moreover, the $s^{50}_{20,w}$ value is quite similar to that previously reported for T7 DNA (10). In contrast the same NH_2OH-treated DNA, sedimented at alkaline pH, has marked variability in sedimentation velocities. In this case, there is a sedimentation velocity difference of 16 between the slowest and most rapidly moving molecules.

As shown in Table 1, at neutral pH the molecular weight of NH_2OH-treated DNA at $s^{50}_{20,w}$ is only slightly less than the accepted molecular weight of 26.4 X 10^6 for native T7

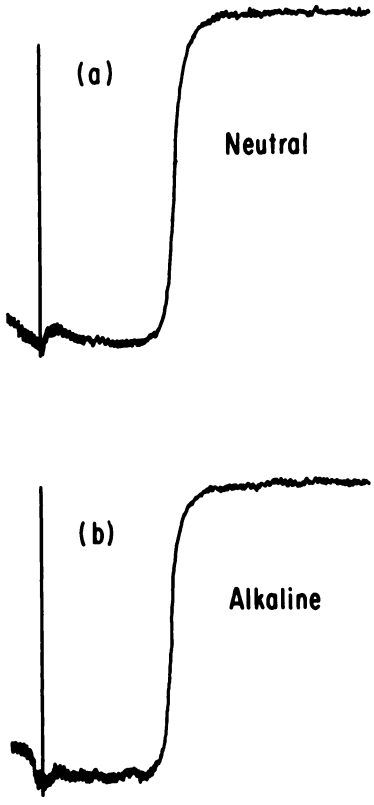

Chart 3. Sedimentation velocity of T7 DNA at neutral (*a*) and alkaline (*b*) pH. Ultraviolet photographs were obtained at 4-min intervals. The profiles shown in this chart were obtained after 8 min sedimentation at 44,700 rpm, as described in "Materials and Methods."

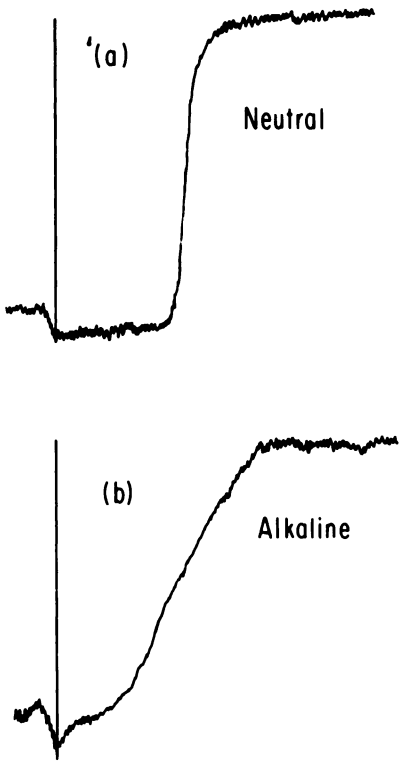

Chart 4. Sedimentation velocity of T7 DNA at neutral (*a*) and alkaline (*b*) pH following treatment with hydroxylamine after incubation in 0.5 M hydroxylamine for 30 min at 37°. DNA was separated from hydroxylamine by chromatography on a Sephadex G-100 column as described in "Materials and Methods." The profiles shown in this chart were obtained as described in Chart 3.

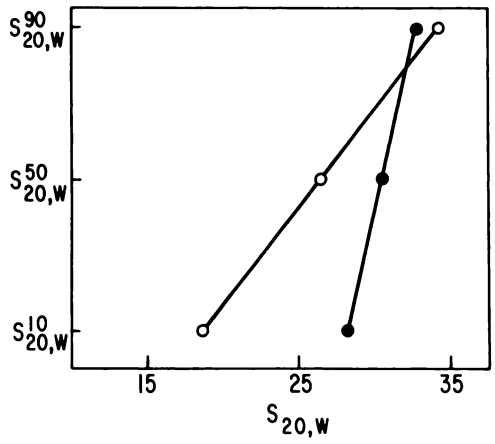

Chart 5. Distribution of sedimentation velocities among DNA molecules treated with hydroxylamine. ○—○, distribution of $s_{20,w}$ values at alkaline pH; ●—●, distribution of $s_{20,w}$ values at neutral pH.

Table 1

$s^0_{20,w}$ measurements and molecular weight assignments for hydroxylamine-treated T7 DNA

Each value for $s^0_{20,w}$ was determined as described in "Materials and Methods." The terms $s^{90}_{20,w}$, $s^{50}_{20,w}$, and $s^{10}_{20,w}$ are used in order to more clearly define a heterogeneous population of DNA molecules, especially at alkaline pH. For example, $s^{90}_{20,w}$ means that at alkaline pH, 90% of the molecules had a calculated $s^0_{20,w}$ value of 34.2 and the median value, or $s^{50}_{20,w}$ was 26.4 at the same pH.

	Neutral pH		Alkaline pH	
	$s^0_{20,w}$	M.W. X 10^6 (double strand)	$s^0_{20,w}$	M.W. X 10^6 (single strand)
$s^{90}_{20,w}$	32.6	26	34.2	12
$s^{50}_{20,w}$	30.4	24	26.4	5
$s^{10}_{20,w}$	28.2	19	18.6	1.8

DNA (10). The molecular weight of single-strand T7 DNA should therefore be approximately 13 X 10^6. Instead, at alkaline pH we find the $s^{50}_{20,w}$ value is only 5 X 10^6 (Table 1). This suggests that almost all the DNA molecules have at least 1 internucleotide scission. This alteration could affect 1 or both of the 2 strands in a DNA double helix, but if each of 2 strands is interrupted, the scission apparently does not involve the same phosphodiester linkage for a single base-pair since sedimentation at neutral pH is not affected by NH_2OH treatment.

If the single-strand scissions produced by hydroxylamine action on T7 DNA create regions with increased affinity for RNA polymerase (*e.g.*, single-strand regions), then greater amounts of polymerase might be required in order to cause maximal retention of the DNA on membrane filters. As shown previously, 1 μg RNA polymerase is sufficient to cause retention of 2 μg ^{3}H-labeled T7 DNA on a membrane filter (5). As shown in Chart 6, 3 times that much polymerase is required in order to reach maximal retention of T7 DNA treated with either salt-free NH_2OH or T7 DNA from which hydroxylamine has been removed by passage over a Sephadex G-100 column (see Chart 1). The observation that, even after removal of hydroxylamine, greater amounts of polymerase are required to bind all DNA on the filter suggests that this effect is not attributable to alteration of RNA polymerase by hydroxylamine.

We demonstrated in Chart 2 that transcription of native T7 DNA is progressively inhibited as a function of incubation time with hydroxylamine. This effect on transcription can be demonstrated in another way. Recently, we have observed that certain forms of damage to DNA molecules, for example, shearing (A. E. Cato, S. B. Crist, and O. W. Jones, manuscript in preparation) or subcritical heating (unpublished results) resulted in transcription with at least 2 different slopes of RNA synthesis when increasing amounts of RNA polymerase are added to a constant amount of altered DNA. In the presence of a constant amount of DNA and increasing quantities of RNA polymerase, there is an initial linear slope of minimal synthesis followed by an abrupt change to a greater amount of synthesis at a certain concentration of RNA polymerase. Usually, the second slope approaches that seen with undamaged DNA. As shown in Chart 7*a*, when freshly prepared, undamaged T7 DNA at a constant concentration is incubated with increasing amounts of RNA polymerase, there is a linear relationship between RNA synthesis and polymerase concentration. In contrast if the same DNA preparation is treated for 7.5 min (Chart 7*b*) or 15 min (Chart 7*c*) with salt-free NH_2OH, we observe an initial small linear slope as reported earlier (A. E. Cato and O. W. Jones, submitted for publication) but in contrast to our previous observations, the second slope, beginning at 1.0 μg RNA polymerase, is only slightly less but certainly not greater than the initial slope. This suggests that hydroxylamine treatment results in damage to DNA which prohibits optimal transcription at any concentration of polymerase.

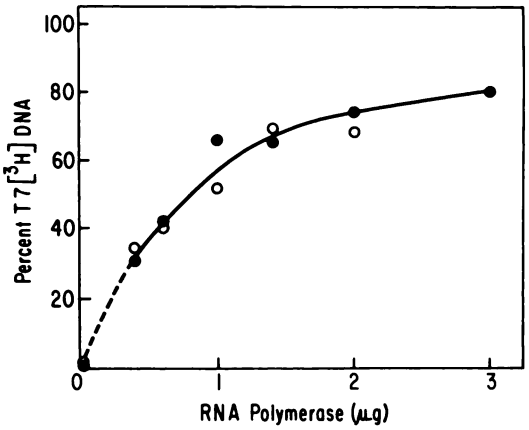

Chart 6. Retention of hydroxylamine-treated T7 DNA on membrane filters in the presence of RNA polymerase. Assays were performed as described in "Materials and Methods"; 11,600 cpm as ^{3}H-labeled T7 DNA represents 100% retention. In the absence of polymerase, 64 cpm are retained on a membrane filter. In each experiment T7 DNA was incubated for 30 min at 37° in 0.5 M salt-free, recrystallized hydroxylamine. •—•, a binding assay with T7 DNA after removal of hydroxylamine by chromatography on a Sephadex G-100 column, ○—○, binding assay in the presence of hydroxylamine.

DISCUSSION

The effect of hydroxylamine on pyrimidine bases in synthetic ribopolynucleotides seems well established (7–9, 13). It also seems reasonable that these changes do alter specifically the template properties of certain synthetic ribopolynucleotides. However, it seems that the mutagen exerts additional damage on native double-helical DNA templates. There is clear evidence that at least 1 effect on native DNA is the production of single-strand scissions. Moreover, it would seem from the heterogeneity of hydroxylamine-treated T7 DNA at alkaline pH (Chart 4*b*) that this damage is extensive. The membrane binding experiment suggests that the result of single-strand scissions in native T7 DNA is the creation of sites on the template which compete for available enzyme, thus limiting the amount of enzyme available for a natural RNA "initiator"

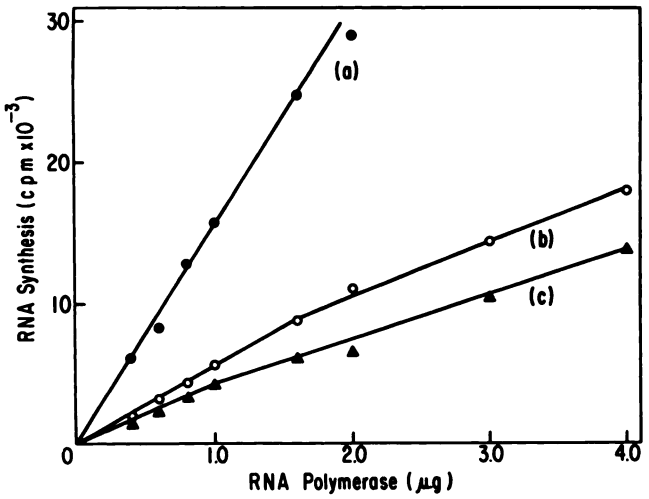

Chart 7. Transcription of T7 DNA with increasing amounts of RNA polymerase. Assays were performed as described in "Materials and Methods" and incubation was 5 min at 37°. Each reaction mixture contained the labeled nucleoside triphosphate, ribocytosyltriphosphate, specific activity 1.7 X 10^7 cpm/μmole and 4 μg T7 DNA. *a*, freshly prepared T7 DNA; *b*, T7 DNA incubated in 0.5 M desalted, recrystallized hydroxylamine for 7.5 min at 37°; *c*, T7 DNA incubated in 0.5 M desalted, recrystallized hydroxylamine for 15 min at 37°.

site or for other DNA molecules. We have shown previously that single-strand regions in DNA molecules, denatured by alkali, are at least 20 times more effective than native DNA in binding RNA polymerase (5, 10). The single-strand scissions caused by hydroxylamine might result in localized regions of denaturation at the internucleotide site of single phosphodiester bond cleavage. Such regions should compete more strongly for available polymerase than other *undamaged* regions on the molecule. However, we also cannot exclude the possibility that the putative RNA polymerase-NH_2OH-treated DNA complex simply does not bind effectively to a membrane filter. If an effect of hydroxylamine on native T7DNA is to create additional attachment sites for polymerase, these sites are not effective in initiating polymerization of RNA molecules. As shown in Chart 7, at all concentrations of polymerase tested, there is less RNA synthesis on a hydroxylamine-treated template compared with untreated DNA.

There is increasing evidence that *in vivo* pyrimidine-rich strands of the DNA double helix are transcribed preferentially by RNA polymerase, although *in vitro* both DNA strands are transcribed (11, 12). It is possible that the single-strand scissions shown here are localized primarily to cytidine-rich sequences in the DNA molecule which ordinarily are part of the binding site for polymerase. Damage to pyrimidines in these regions results in increased susceptibility to internucleotide scissions. RNA polymerase has greater affinity for these damaged, perhaps single-strand regions, but polymerization of nucleotides is inhibited, perhaps because of structural damage to the pyrimidines. What does seem certain is that the effect of hydroxylamine on native DNA templates is not restricted to alteration of individual pyrimidine bases.

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