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N^4-Aminocytidine, a nucleoside analog that has an exceptionally high mutagenic activity

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ABSTRACT

The reaction of cytidine with hydrazine to give N^4-aminocytidine was greatly promoted by addition of a less-than-stoichiometric amount of bisulfite, and the product was isolated in a good yield. N^4-Aminocytidine was strongly mutagenic to bacteria (Salmonella typhimurium TA100 and TA1535, and E. coli WP2 uvrA) and to phage (φX174 am3). The activity did not require the presence of mammalian microsomal fraction in the system. The mutagenic potency of N^4-aminocytidine in these systems was two orders of magnitude greater than that of N^4-amino-2'-deoxycytidine, and more than two orders of magnitude greater than that of N^4-hydroxycytidine. The greater activity of the riboside than the deoxyriboside was ascribed to the lack of deoxycytidine kinase in these cells. This compound may be useful as a powerful mutagen to induce a transition mutation in microorganisms.

INTRODUCTION

Some nucleoside analogs and base analogs are known to be mutagenic. 5-Bromouracil and 2-aminopurine are typical examples, and their actions have been extensively investigated for understanding mutational and replicational processes. However, compared to those mutagens which chemically react with DNA and modify the bases, these base-analog mutagens are weak in activity. Recently, N^4-hydroxycytidine, 6-hydroxyamino-2-aminopurine, and N^4-amino-2'-deoxycytidine were found to be stronger mutagens to bacteria and phages than previously known compounds of this type (1-5). The mutagenicity of N^4-hydroxycytidine has been studied in depth by Janion and coworkers (1,3,4). N^4-Hydroxycytidine 5'-triphosphate, which should be the product formed in bacteria from N^4-hydroxydeoxycytidine, has been extensively used as a precursor of DNA synthesis in vitro to mediate site-directed mutagenesis (6-8).

We have been studying the bisulfite-catalyzed transamination of cytidine (9-11). As one of the products of those reactions, N^4-aminocytidine was assayed for its mutagenic activity in bacteria and in phage. Surprisingly, the activity was found to be much greater than that of either N^4-hydroxycytidine or N^4-aminodeoxycytidine. In this report, we describe the mutagen-

icity of N^4-aminocytidine and discuss the mechanism of the mutagenesis.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhimurium stains were gifts from Dr. B. N. Ames (Univ. of California)(12). Escherichia coli WP2 strains were kindly offered by Dr. M. Ishizawa (Univ. of Kyushu)(13). Bacteriophage φX174 am3 and its host E. coli strains were generous gifts from Dr. M. Sekiguchi (Univ. of Kyushu).

Preparation of nucleoside analogs

For preparation of N^4-aminocytidine, the transamination promoted by a less-than-stoichiometric amount of bisulfite was used. This reaction was carried out in a phosphate-buffered solution at pH 7. It is known that phosphate ion facilitates the desulfonation of 5,6-dihydro-N^4-aminocytidine-6-sulfonate to restore the 5,6-double bond (ref. 11 and unpublished work). Thus, cytidine, 100 mg, in 1 ml solution containing 4 M hydrazine, 0.1 M sodium bisulfite, 0.1 M sodium phosphate, of which pH had been adjusted to 7 with HCl, was allowed to react at 60° for 4 hr. The salts were then precipitated by addition of 9 ml cold ethanol. The mixture was allowed to stand in a freezer at - 20° for 1 hr, and was centrifuged. The supernatant was evaporated under reduced pressure forming a thick syrup. The syrup was treated with ethanol to give a crude sample of N^4-aminocytidine as a semi solid: yield, 70 % (and 66 % in another run) as based on UV absorbance. In a separate experiment, the molar extinction coefficient of N^4-aminocytidine 5'-phosphate was estimated to be 10,900 at 274 nm (λ_{max} at pH 7) based on the phosphorus content of the sample.

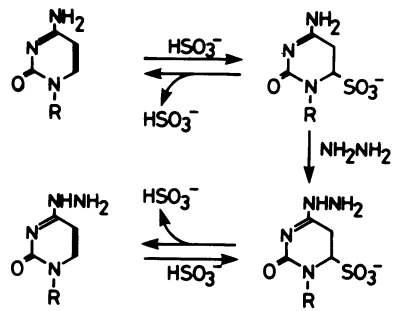
A sample of crude N^4-aminodeoxycytidine was prepared from 2'-deoxycytidine by the same procedure (yields, 84 % and 98 % in two experiments).

The N^4-aminocytidine and N^4-aminodeoxycytidine samples thus obtained were further purified by high pressure liquid chromatography (HPLC) using a Lichrosorb RP-18 (Merck) column. The eluting solvent used was 0.05 M HCOOH-NH_3, pH 4.10, and the flow rate was 1 ml/min. The peaks were detected by monitoring $A_{254\ nm}$. The desired compounds were eluted as major peaks in the chromatograms. The pooled fractions were lyophilized and the residue obtained was dissolved in water to prepare stock solutions for the mutagenicity assay. The solutions were stored frozen until they were used.

N^4-Hydroxycytidine was prepared and purified as reported (4).

Each of these three nucleoside samples prepared was checked for purity by rechromatography on the HPLC column, and gave a single peak.

Scheme 1



For the time-course study of the N^4-aminocytidine formation, cytidine was treated with a mixture of hydrazine, bisulfite, and phosphate in the way described above. Aliquots were withdrawn and diluted, and the samples equivalent to 0.2 µl of the reaction mixture (corresponding to 80 nmole of nucleoside) were analyzed by HPLC for the product distribution, and those equivalent to 0.1 µl were assayed for mutagenicity on S. typhimurium TA1535 without addition of S9 (see below for the mutation assay). The conditions for HPLC were the same as those used for the preparative experiments. The retention time was 6.8 min for N^4-aminocytidine, 8.0 min for cytidine, and 11.2 min for uridine (see Fig. 2).

Mutation assay

Procedures previously described in the literature were used for the assay on S. typhimurium (14) and E. coli (13). The 9,000 x g supernatant of liver homogenate (S9) used in these assays was prepared from rats induced with polychlorinated biphenyl.

The reversion frequency of φX174 am3 was measured as follows. E. coli HF4714 (su^+) was cultured in φXC medium. This medium was similar in composition to TPG3A described by Sinsheimer (15), but it contained three times higher concentration of casamino acid and no pyruvate. The E. coli was grown at 37° to 2-3 x 10^8 cells/ml. To this were added φX174 am3 at m.o.i. 30, and the mutagen. Then the culture was incubated at 32° for 5.5 hr. EDTA was added to 0.5 mM and the mixture was allowed to stand at 4° overnight. The lysate thus obtained was titrated with E. coli HF4714 (su^+) and with E. coli CI. The mutation frequency was obtained as [PFU assayed on E. coli CI]/[PFU assayed on HF4714].

In all the mutation assays, each datum represents a mean in two plates. For each value, the background revertant score had been subtracted.

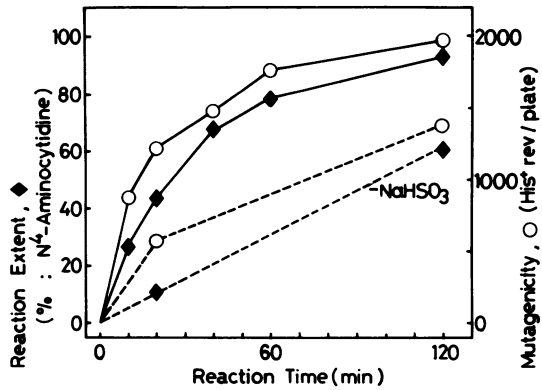


Fig. 1. Mutagen formation as a function of N^4-aminocytidine formation in the reaction between cytidine and hydrazine-bisulfite. The reaction conditions are given in the text. For the estimation of reaction extents, see Fig. 2. The mutagenicity was assayed on S. typhimurium TA1535 in the absence of S9. In the control experiments (dotted lines), bisulfite was omitted from the reaction mixture.

RESULTS

As Fig. 1 shows, the transamination of cytidine with hydrazine to give N^4-aminocytidine, mediated by bisulfite, proceeded smoothly to near completion. The molar-ratio of the reagents, cytidine/hydrazine/bisulfite, present in the reaction mixture was 1/10/0.25. This small amount of bisulfite was very effective in promoting the reaction: in the absence of bisulfite, the transamination was much slower. In the HPLC-analysis of the reaction mixture (Fig. 2), only N^4 aminocytidine and cytidine were the major nucleosidic components detectable. It should be noted that no uridine was detected. The small peaks that emerged very early in the HPLC (6 % of the total A_{254} value in the 120 min-reaction) were probably those of the bisulfite adducts of the product. Fig. 1 also shows that the mutagenicity, as measured on S. typhimurium TA1535 in the absence of S9, increased as a function of the reaction extent. The mutagenic activity at time zero was 10 revertants/plate. This indicated that the reagents themselves, i.e. cytidine, bisulfite and hydrazine, had no mutagenic activity in this system.

The mutagenicity of N^4-aminocytidine was tested with a set of S. typhimurium strains. The results are shown in Fig. 3 and Table 1. N^4-Aminocytidine showed a strong mutagenic activity. It was mutagenic to the hisG-46 bearing strains TA100 and TA1535 both in the presence and absence of S9. No reversions were observed in TA98, TA1537 or TA1538, which are frameshift-mutagen

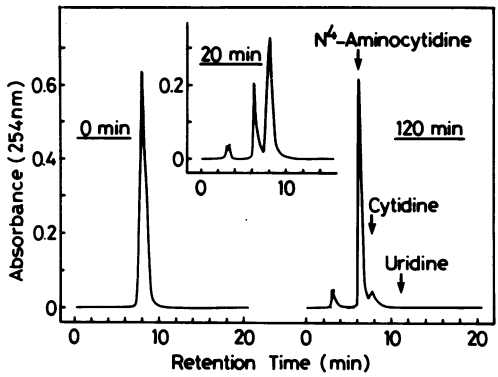


Fig. 2. High pressure liquid chromatographic analysis of the reaction between cytidine and hydrazine-bisulfite. The reaction extent, which is shown in Fig. 1, represents the fraction, in percent, of the area corresponding to N^4-aminocytidine in the sum of the total peak areas. The column size was 4.6 x 250 mm.

detectors. N^4-Aminodeoxycytidine was mutagenic to TA100 and TA1535, but it was much less potent than N^4-aminocytidine. N^4-Hydroxycytidine exhibited no significant mutagenicity to these Salmonella strains. The mutagenic activity of N-nitrosomethylurea, which was tested as a positive control, was consistent with the activity reported in the literature (16).

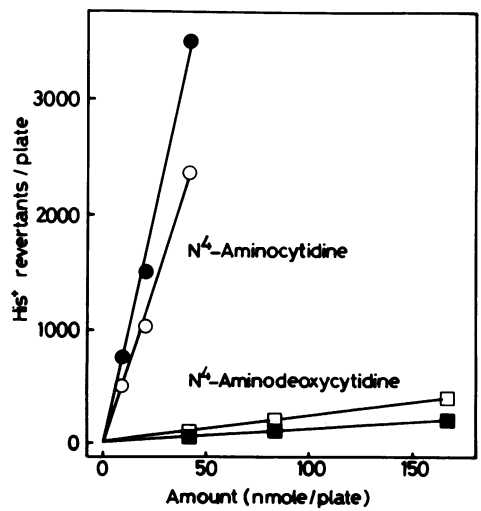


Fig. 3. Mutagenicity of N^4-aminocytidine and N^4-amino-2'-deoxycytidine on S. typhimurium TA100. Solid symbols, +S9; open symbols, -S9.

Table 1. Mutagenicity of N^4-aminocytidine, N^4-amino-2'-deoxycytidine and N^4-hydroxycytidine on bacteria

Bacteria and marker	Mutagenicity (number of revertants/nmol) [a]						
	N^4-Aminocytidine		N^4-Amino-2'-deoxy-cytidine		N^4-Hydroxycytidine		N-Nitrosomethylurea
	-S9	+S9	-S9	+S9	-S9	+S9	-S9
S. typhimurium ($his^- \rightarrow his^+$)							
TA100	60	89	2.4	1.0	0	0	6.5 (4.4 [b])
TA1535	80	116	3.0	1.2	0	0	- [c]
TA98	0	0	0	0	0	0	-
TA1537	0	0	0	0	0	0	-
TA1538	0	0	0	0	0	0	-
E. coli ($trp^- \rightarrow trp^+$)							
WP2 *uvrA*	155	185	3.5	1.9	0.5	0.5	-
WP2 *uvrA*/pKM101	348	396	-	-	-	-	-
WP2	59	54	-	-	-	-	-
WP2 pKM101	61	78	-	-	-	-	-

a: The values were calculated from the linear portions of the dose-response curves.
b: A value reported in the literature (16).
c: Not done.

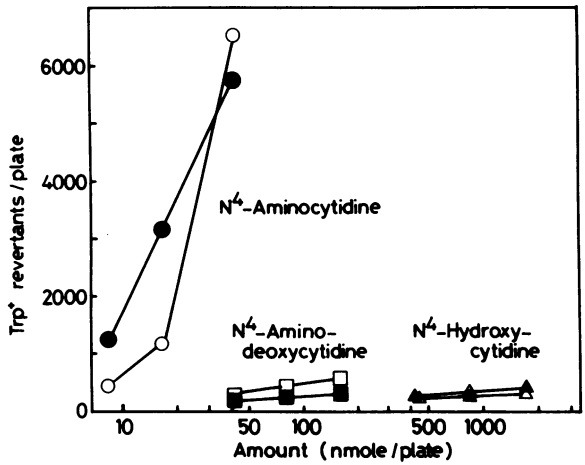


Fig. 4. Mutagenicity of cytidine analogs on E. coli WP2 uvrA. Solid symbols, +S9; open symbols, -S9.

N^4-Aminocytidine was also strongly mutagenic to E. coli WP2 (Fig. 4 and Table 1). Again, N^4-aminodeoxycytidine was much less mutagenic than N^4-aminocytidine. N^4-Hydroxycytidine showed mutagenicity to the E. coli which was consistent with the observation in the literature (4): the mutation frequency found in the present experiment was equivalent to the value reported. In E. coli WP2, both the absence of the uvr gene and the presence of plasmid pKM101 were found to enhance the activity of N^4-aminocytidine. This contrasts to the activity in S. typhimurium where the presence of pKM101 (TA100) or its absence

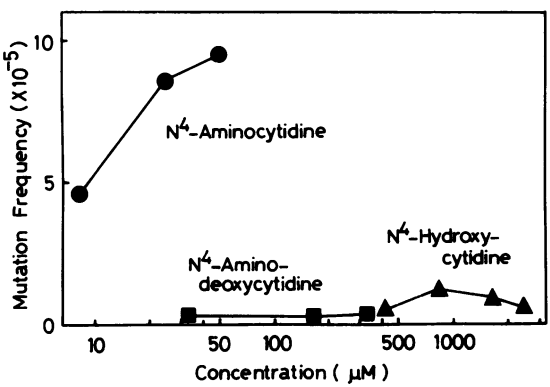


Fig. 5. Reversion of φX174 am3 by cytidine analogs.

(TA1535) did not give different response to N^4-aminocytidine.

N^4-Aminocytidine was also mutagenic to bacteriophage φX174. When φX174 am3, an amber mutant. was grown in E. coli in the presence of N^4-aminocytidine in the culture medium, reversion to wild type took place. As shown in Fig. 5, N^4-aminocytidine showed a very strong activity. In contrast, N^4-aminodeoxycytidine was inactive within the concentration range examined. N^4-Hydroxycytidine was mutagenic, but the activity was much smaller than N^4-aminocytidine. When φX174 am3 phages were treated directly with 20 mM or 50 mM N^4-aminocytidine in the absence of the host bacteria, no induction of mutation was observed.

DISCUSSION

In the bisulfite-mediated reactions of cytosine compounds, bisulfite adds across the 5,6-double bond of the pyrimidine ring to make the C^4-amino group susceptible to nucleophilic agents (17,18). The pyrimidine compounds in this reaction, i.e. the cytosine and the transaminated product, bind with bisulfite to form the 5,6-dihydropyrimidine-6-sulfonates. Under conditions where the bisulfite adduct of the pyrimidines can liberate bisulfite in a reversible process, it may be expected that a less-than-stoichiometric amount of bisulfite is sufficient to promote transamination of cytosine at the N^4-position. This was demonstrated to be the case in the present experiments for the formation of N^4-aminocytidine and N^4-aminodeoxycytidine. Thus, the transamination proceeded smoothly, and the bisulfite adduct of N^4-aminocytidine formed in the reaction mixture was only a small fraction, if any, of the total nucleosidic components. In the previously reported methods (9,10,17,19,20), a large excess of bisulfite was used for this type of reaction, in which the bisulfite adducts of the products should first be isolated and then the 5,6-double bond must be generated from the adducts.

As a nucleoside analog, N^4-aminocytidine has an exceptionally strong mutagenicity. Its potency in S. typhimurium TA1535 and TA100 and in E. coli WP2 uvrA is two orders of magnitude greater than that of N^4-aminodeoxycytidine, and more than two orders of magnitude greater than that of N^4-hydroxycytidine. It should be noted that N^4-aminodeoxycytidine (5) and N^4-hydroxycytidine (3) have been regarded as very potent mutagenic compounds among the base analogs. The mutagenic activity of N^4-aminocytidine is more than one order of magnitude greater than that of the direct mutagen N-nitrosomethylurea, as tested in the Ames' Salmonella system (Table 1). Also, N^4-aminocytidine appears to have a broad spectrum: in a test with Bacillus subtilis, N^4-aminocytidine was again

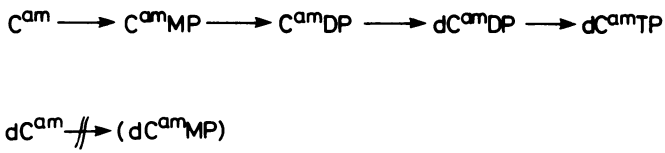


Fig. 6. A mechanism to explain the stronger activity of the riboside than the deoxyriboside.

very mutagenic (unpublished work). It would be of interest to examine the mutagenicity of N^4-aminocytidine in mammalian cells.

A plausible mechanism for the mutagenesis is shown in Figs. 6 and 7. It can be expected that N^4-aminocytidine is metabolized in cells in the same way as cytidine, and as a result, N^4-aminodeoxycytidine 5'-triphosphate is formed, which in turn can be used as a substrate for DNA synthesis. The inactivity of N^4-aminodeoxycytidine can be explained in terms of the lack of deoxycytidine kinase in S. typhimurium and E. coli (21). Similar observations was made by Janion (4) for N^4-hydroxycytosine derivatives, i.e. the riboside was more mutagenic than the deoxyriboside.

N^4-Aminodeoxycytidine triphosphate would be incorporated into DNA mostly in place of dCTP. At a low, but significant rate, the N^4-aminodeoxycytidine triphosphate might be used as a substitute of dTTP (step 1 in Fig. 7). In subsequent replication (step 2), the major event would be incorporation of guanine in the complementary position, and the net result would be an AT-to-GC transition. N^4-Aminocytosine substitution at the site of cytosine in DNA (step 1) should be mutagenic, too. In this case, incorporation of adenine in step 2 would lead to a GC-to-AT transition. Previous observation that the

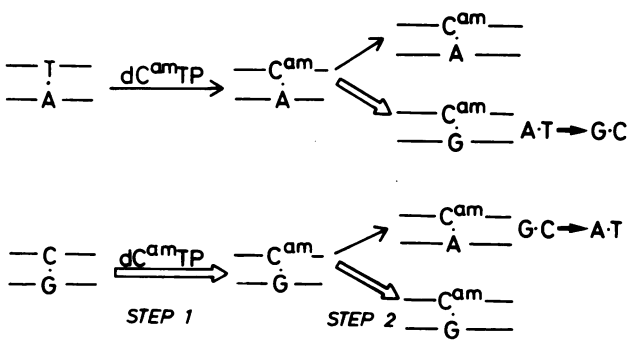


Fig. 7. Mechanisms of N^4-aminocytosine-mediated mutagenesis.

treatment of λ phage with a mixture of bisulfite and hydrazine caused mutation (11) would correspond to this mechanism.

The reversion of phage φX174 am3 to wild type must be a result of transition AT-to-GC, if it is a transition but not a transversion. A GC-to-AT transition in the amber codon would produce UAA which is again a nonsense codon.

Because N^4-aminocytidine had an exceptionally high mutagenic activity, attention should be directed to the possibility of a direct chemical interaction between N^4-aminocytidine and DNA. Such an interaction is unlikely because a direct treatment of φX174 with N^4-aminocytidine did not yield mutation. In line with this, N^4-aminodeoxycytidine showed only poor mutagenicity, in spite of the fact that the N^4-amino group of N^4-aminodeoxycytidine is as reactive as that of N^4-aminocytidine toward various reagents.

In conclusion, N^4-aminocytidine would be a useful nucleoside analog in studies of mutation. An obvious application would be to use it as a powerful agent to induce a transition mutation in microorganisms.

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