The Effects of Hydroxylamine on the Template Properties of Polycytidylic Acid

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SUMMARY

Treatment of polycytidylic acid with hydroxylamine produces an inactivation of its template properties as measured by incorporation of guanosine 5'-phosphate. Partial recovery of the guanosine 5'-phosphate incorporation can be obtained by the addition of adenosine 5'-triphosphate, but not cytidine 5'-triphosphate or uridine 5'-triphosphate. Nearest neighbor studies showed that the adenosine 5'-phosphate was being incorporated exclusively next to guanylate; there was none next to adenyate. These results suggest that the hydroxylamine produces in the polycytidylic acid a species by which adenosine 5'-phosphate is incorporated in place of guanosine 5'-phosphate, and then a second species is produced which will not pair with any of the normal bases.

Hydroxylamine has been shown to have a strong mutagenic and inactivating effect upon bacteriophages (1-4). An examination of the reactivity of hydroxylamine with the purines and pyrimidines of deoxyribonucleic acid and of ribonucleic acid established (a) that the purines were unreactive in the presence of hydroxylamine, and (b) that of the pyrimidines, cytosine was the most reactive (5-9).

Although there has been disagreement as to the intermediate formed in the reaction between cytosine and hydroxylamine (5-10), these biological and chemical studies have strongly suggested that mutagenesis caused by hydroxylamine was the result of the formation of an altered cytosine which then paired with adenine instead of guanine during DNA replication. The effects of hydroxylamine upon DNA was thus to induce the base pair transition of guanine-cytosine to adenine-thymine (6).

The recent work of Kotaka and Baldwin (11) and of Ono, Wilson, and Grossman (12) with model polymerase systems suggested an approach to the study of mutagenesis. The former (11) studied the effect of nitrous acid upon the template activity of poly C copolymer with a purified DNA polymerase, and the latter (12) treated polycytidylic acid with ultraviolet light and studied the coding properties of the altered polymer with an RNA polymerase.

In this study we used an RNA polymerase purified from Micrococcus lysodeikticus (13) to investigate the effect of hydroxylamine on the template properties of polycytidylic acid.

EXPERIMENTAL PROCEDURE

M. lysodeikticus cells and polycytidylic acid were obtained from Miles Chemical. The poly C was purified by phenol extraction followed by reprecipitation with isopropyl alcohol, and then dialyzed against 0.2 M NaCl overnight prior to use. All 14C- and 3H-labeled nucleotides were obtained from Schwarz BioResearch. 32P-Labeled triphosphates were purchased from International Chemical and Nuclear Corporation.

Preparation of Enzyme—The RNA polymerase was isolated from dried M. lysodeikticus cells by Procedure A of Nakamoto, Fox, and Weiss (13). The enzyme obtained was stored in 50% glycerol at -15°.

Treatment of Polycytidylic Acid by Hydroxylamine—Hydroxylamine hydrochloride was dissolved in 0.6 M sodium succinate buffer and pH was adjusted to 6.2; then poly C and subsequently water were added to give a final concentration of 750 μg of poly C per ml and 0.1 M sodium succinate buffer. (The final hydroxylamine concentration was varied from 0.1 μl to 1.0 μl.) A pH of 6.2 was selected for most of these experiments since it was found (a) that this was the pH at which cytosine reacted optimally. The reaction mixture was incubated at 20°, 30°, or 40°. Aliquots were removed at hourly intervals for the determination of the template activity of poly C, for optical density, and for acid treatment as described below.

Template Activity of Poly C—The assay used is that of Fox, Robinson, Haselkorn, and Weiss (14). Each tube contained 30 μmoles of Tris buffer (pH 7.8), 1.2 μmoles of MnCl2, 20 μg of poly C (or hydroxylamine-treated poly C), guanosine 5'-triphosphate-3H, and enzyme in a final volume of 0.3 ml. Control experiments were performed initially in the absence of either Mn++ or poly C. After incubation for 20 min at 30°, the samples were chilled, and 0.1 ml of 50% TCA was added. After standing for 3 min, 2 ml of 5% TCA were added. The samples were collected on a Millipore filter and washed twice more with 2 ml of 5% TCA. The filters were dried, and counted in a windowless gas flow counter with a counting efficiency of approximately 20%. The controls (minus poly C) always contained less than 0.5% of the radioactivity found in the samples.
Nearest Neighbor Studies—Experiments were performed with GTP-α-[32P] or ATP-α-[32P]. The incubation was performed in a volume of 0.6 ml, and 0.3-ml aliquots were precipitated, washed, and plated, and the radioactivity was then determined. Another 0.3-ml aliquot from the same incubation mixture was removed, 500 μg of yeast RNA were added as carrier followed by 0.1 ml of 1 M sodium pyrophosphate, 0.1 ml of EDTA (pH 7.5), and 0.1 ml of 50% TCA, and the mixture was allowed to stand for 10 min. Then 2 ml of 5% TCA were added, and the tubes were centrifuged. The pellet was washed twice with 2 ml of 5% TCA, once with ether, and allowed to dry in air. The pellets were incubated with 0.2 ml of 0.3 M KOH for 18 hours at 37°C. The samples were chilled in ice and the pH was adjusted to 3.0 with 10 N HClO₄, and the samples were centrifuged to remove KC104. The samples were placed on Whatman No. 3MM paper and subjected to electrophoresis in 0.05 M ammonium formate at pH 3.5 and 14 mv per inch for 5 hours. The paper was removed, air-dried, and scanned with an ultraviolet light. The area of the paper containing the absorbing nucleotides was marked, cut out, glued to metal planchets, and counted directly. Control samples did not contain poly C. The amount of radioactivity found in the 3'(2')-nucleotide (Gp, Ap, Cp, and Up) was a measure of the transfer of label from the 5'-nucleotide (GTP-α-[32P] or ATP-α-[32P]). The percentage of recovery of radioactivity following electrophoresis was based on the amount found in the 0.3-ml aliquot.

Absorbance Determinations—Aliquots of the hydroxylamine-poly C reaction mixture were removed at intervals, and the decrease in optical density at 269 nm was determined in a Beckman DU spectrophotometer. An appropriate blank containing buffer and hydroxylamine at the same concentration was used. The reaction was also followed continuously at 30°C in a Gilford multiple sample absorbance recorder.

Acid Treatment of Hydroxylamine-Poly C Reaction Product—During the reaction of hydroxylamine with poly C, in addition to the aliquots removed at hourly intervals for enzymatic assay, 1-ml samples were also removed. These were placed on Sephadex G-100 that had been equilibrated with 0.2 M NaCl. The samples were eluted with the same concentration of NaCl, and the optical density of the eluate at 225 nm and 269 nm was determined with a Gilford multiple absorbance recorder.

RESULTS

The stability of poly C to the conditions of the preincubation procedure was examined. Fig. 1 shows the effect of treatment of poly C at pH 6.2 and 30°C in the absence of hydroxylamine on the template activity with respect to both GMP and AMP incorporation. There was essentially no inactivation of the template activity of poly C as measured by GMP incorporation at this pH; furthermore, this template activity was unaffected by the addition of both ATP and GTP. When ATP was used with unlabeled GTP and the preincubated poly C, the amount of incorporation of AMP relative to that of GMP was negligible (less than 0.1%).

Preincubation of poly C with 0.1 M hydroxylamine for 6 hours reduces the template activity of the poly C to 75% of the control value (Fig. 2), as measured by GMP incorporation. There was essentially no inactivation of the template activity of poly C as measured by GMP incorporation at this pH; furthermore, this template activity was unaffected by the addition of both ATP and GTP. When ATP was used with unlabeled GTP and the preincubated poly C, the amount of incorporation of AMP relative to that of GMP was negligible (less than 0.1%).

Preincubation of poly C with 0.1 M hydroxylamine for 6 hours reduces the template activity of the poly C to 75% of the control value (Fig. 2), as measured by GMP incorporation. The addition of ATP (but not CTP or UTP) to the treated poly C only partially restores the activity. It is possible that deamination of the poly C is involved here, but, in addition, it is apparent either that there is an additional chemical product formed which is not complementary to any of the normal purine or pyrimidine bases, or else that there is depolymerization or glycosidic bond fission, or a combination of these events.

Fig. 3 shows the effect of pretreatment of poly C with various concentrations of hydroxylamine on the template activity of poly C with respect to GMP incorporation in the absence of
ATP. It appears that the inactivation of poly C is a linear function of the hydroxylamine concentration over the lower concentrations tested. This suggests that the inactivation is a single chemical event. The inactivation of the template can also be accelerated at a constant concentration of hydroxylamine by increasing the temperature. For example, the percentage of inactivation for the 1st hour is 50% when the polymer is preincubated at 30°C in 1 M NH₂OH, and 70% when the polymer is preincubated at 40°C in 1 M NH₂OH.

Fig. 4 shows a series of experiments in which ATP⁻¹⁴C was used with unlabeled GTP and hydroxylamine-treated poly C. The data are reported as the percentage of AMP incorporated into the polymer, poly AG. This was done by simultaneously measuring GMP⁻¹⁴C incorporation, which gives a measure of the amount of polymer being synthesized. The amount of AMP⁻¹⁴C incorporated was then divided by the sum of both GMP⁻¹⁴C incorporated and the AMP⁻¹⁴C for the time period in question. The number of micromicromoles of AMP⁻¹⁴C at zero time was subtracted from the values obtained during other time periods prior to the percentage calculation. There was always a small amount of AMP incorporated at zero time, and this value remained constant in the absence of GTP during the treatment period. It can be seen in Fig. 4 that in the absence of hydroxylamine the percentage of incorporation of AMP is very small, amounting to less than 0.1%. At a concentration of 0.2 M NH₄OH and 30°C, the percentage of AMP incorporated is 1% for the entire 6-hour period, and at 0.5 M NH₄OH and 30°C, the percentage of AMP incorporated is approximately 2%. The percentage of incorporation of AMP in the presence of GTP on a template of poly C treated with 1 M NH₄OH at 30°C is very similar to that observed when a concentration of 0.2 M was used. But when the polymer was treated with 1 M NH₄OH at 40°C and the incorporation of AMP⁻¹⁴C was measured (in the presence of GTP), the percentage incorporated increased to a maximum of 0.6% in 3 hours, and then rapidly decreased to zero. There appears to be a slight break in all curves at approximately 3 hours of treatment. This was observed at different concentrations of hydroxylamine and at different treatment temperatures. However, the over-all incorporation of AMP into the polymer was affected both by the temperature and by the concentration of the hydroxylamine during pretreatment.

The fact that the percentage of AMP incorporated on a poly C template treated with 1 M NH₂OH is lower than for one treated with 0.5 M NH₂OH at 30°C, and that there is a rapid decrease in the percentage of AMP incorporated with a concentration of 1 M at 40°C, is evidence that as the reaction proceeds, products which are not complementary to the normal purines or pyrimidines are formed. Fig. 4 also shows that the percentage of incorporation is not linear. Since the maximum percentage of incorporation was obtained at 0.5 M and 30°C, the reaction between hydroxylamine and cytosine at this concentration and temperature was examined more thoroughly.

Fig. 3 shows the effect of hydroxylamine treatment at 0.5 M, pH 6.2, and 30°C on the template activity of poly C with respect to GMP incorporation. The rate of loss of GMP incorporating activity is rapid at first and then decreases. When treatment with NH₂OH is continued for longer periods, the activity approaches zero. A 6-hour treatment at 30°C and the presence of 0.5 M NH₂OH caused a 75% inactivation of poly C template activity. A 6-hour treatment with 1 M NH₂OH at the same temperature caused a 95% decrease in the template activity of the polymer. Fig. 5 also shows that the GMP incorporation is unaffected by the presence of CTP. There is an inhibition of GMP incorporation in the presence of UTP. On the other hand, ATP causes a partial restoration of GMP-incorporating activity. This partial restoration of GMP incorporation by ATP was observed at each of the temperatures, hydroxylamine concentra-
FIG. 5. Effect of added triphosphate upon GMP-\(^{14}\)C incorporation with hydroxylamine-treated poly C. Incubation mixtures were the same as in Fig. 2.

When the experiment was performed with ATP-\(^{14}\)C and GTP following treatment of the poly C with 0.5 M NH\(_2\)OH at 30°, the absolute amount of AMP incorporated increased with longer periods of preincubation up to a maximum of 5 hours and then decreased. This is shown in Fig. 6. The percentage of incorporation is shown in Fig. 4. For comparison we note that with pretreatment of the polymer at 20° with 1 M NH\(_2\)OH, the curve reached a maximum at 6 hours; at 30° with 1 M NH\(_2\)OH the maximum incorporation for AMP was 4 hours, and with 1 M NH\(_2\)OH at 40° it was 2 hours. When UTP-\(^{14}\)C or GTP-\(^{14}\)C was substituted for ATP-\(^{14}\)C, there was a small amount of incorporation at zero time which decreased to zero at all other time periods.

In order to determine whether the AMP was being incorporated into a polymer made up of AG or was being attached to the end, nearest neighbor studies were performed with either ATP-\(\alpha\)-\(^{32}\)P or GTP-\(\alpha\)-\(^{32}\)P. In the case of the former, unlabeled GTP was added, and in the latter, unlabeled ATP. The triphosphate with labeled \(^{32}\)P attached to position 5' will be incorporated through a 3'-hydroxyl group. After alkaline hydrolysis which produces 2'- or 3'-nucleotides, the \(^{32}\)P will no longer be attached to the original incorporated nucleotide but to its neighbor. Thus, in the case of ATP-\(\alpha\)-\(^{32}\)P, this is a measure of the internucleotide links of the copolymer, poly AG. In the case of GTP-\(\alpha\)-\(^{32}\)P, it is a measure of the extension of the polymer beyond the incorporated adenylic acid in the guanylic acid polymer.

With a template of poly C preincubated with 0.5 M NH\(_2\)OH at 30° for the usual periods, the amount of GMP-\(\alpha\)-\(^{32}\)P incorporated was measured in the presence and absence of ATP. The decrease in incorporation showed the same kinetic pattern as with the \(^{14}\)C-labeled compound, and the addition of unlabeled ATP partially restored the inactivation of the poly C. When unlabeled GTP and ATP-\(\alpha\)-\(^{32}\)P were used, the amount of incorporation of AMP-\(\alpha\)-\(^{32}\)P increased to a maximum at 5 hours.
and then decreased. Fig. 7 shows the percentage of AMP incorporated into the polymer. The percentage incorporated also drops after 5 hours. Fig. 7 also shows the amount of GpA residues (from ATP-\(\alpha^{-32}P\)) found after nearest neighbor studies. The data are plotted as the percentage of GpA residues (relative to GpG) incorporated into the polymer. The curve is very similar to the AMP incorporation. The amount of ApA residues (adenine incorporated next to adenine) was negligible. These ApA residues are a measure of poly A synthesis and the lack of labeling clearly indicates the absence of such synthesis. The data on poly A synthesis reported here are in contrast to those of Brown and Phillips (10) which showed extensive poly A synthesis under the conditions used in their experiments.

In another experiment GTP-\(\alpha^{-32}P\) was used, and the amount of Gp and Ap residues was determined. This gave the amount of incorporation of guanine next to guanine (Gp) and next to adenine (Ap). At zero time, there was a small amount of ApG residue which increased up to 6 hours and then decreased at 6 hours. Fig. 8 shows these data. The amount of GpG residues followed a pattern similar to GMP incorporation.

In the chemical studies cited earlier (7, 10), it was established that when the final product of the reaction between hydroxylamine and cytosine is heated at pH 4.0, the double bond is restored and the product is 4-(hydroxyamino)cytosine. An attempt was made to determine the effect upon the template properties of the polymer. Based on the data reported here, the first being a necessary precursor to the second and the other causing replication error. The replication error is represented by the abnormal incorporation of AMP instead of GMP on the hydroxylamine-treated poly C template. This abnormal incorporation of AMP was substantiated by the nearest neighbor studies, which showed that all the AMP was incorporated next to guanine and none next to adenine. The inactivation was represented by a decrease in GMP incorporation on the altered poly C template in the presence of AMP.

It had been previously shown that the reaction between cytosine and hydroxylamine causes a decrease in absorbance at 269 m\(\mu\) as a result of the addition of NH\(_2\)OH across the 5,6-double bond. In the earlier stages of the work reported here, an attempt was made to correlate absorbance changes in the poly C due to hydroxylamine reaction with results from polymerase assay. In the first 6 hours of the treatment of poly C with 0.5 m hydroxylamine at pH 6.2 and 30\(^\circ\), the incorporation of GMP decreased by 75%; however, there was no detectable decrease in ultraviolet absorbance. If the poly C was allowed to react for longer periods, there was a detectable decrease in absorbance. If the concentration of hydroxylamine or the temperature was increased, then a decrease in absorbance at 269 m\(\mu\) could be detected in the first 6 hours. For example, at 40\(^\circ\) and 1 m NH\(_2\)OH, at the end of 6 hours when GMP incorporation had decreased 98\%, there was a 28\% decrease in absorbance. However, in the 1st hour of this experiment, the change in absorbance was 2.4\% and the decrease in incorporation was 70\%. This lack of correlation between decrease in absorbance and decrease in GMP incorporation might be due to a change in the secondary structure of the poly C or to depolymerization. It is apparent from the data presented that only a portion of the GMP incorporation is restored by the addition of ATP, and the question is whether this further decrease in GMP incorporation is the result of (a) two sequential chemical events occurring at the same cytosine residue, the first being a necessary precursor to the second, or (b) separate and unrelated chemical events.

The final chemical product of the reaction between cytosine and hydroxylamine has been established previously by other workers as 5,6-dihydro-6-hydroxylaminocytosine-\(N^\alpha\)-(hydroxylaminocytosine), Compound b (Fig. 9) (5-7, 9). Recently Brown and Phillips (10) have reported a lag in the production of Compound b in a poly C polymer treated with hydroxylamine. Based on this experiment and also previous work, they consider that Compound b is a stable intermediate.
Compound a (5,6-dihydro-6-(hydroxylamino)cytosine) is the most likely intermediate. The other structure important for discussion is that of Compound c (N^6-(hydroxylamino)cytosine), which is obtained from Compound b by treating this compound at pH 7.

It has been speculated (15) that Compound a was the species causing the replication error, and Compound b was the inactivating one. This followed from the work of Ono, Wilson, and Grossman (12), in which an explanation was proposed concerning the abnormal base pairing of the hydrate of cytosine. The hypothesis was that 5,6-dihydrocytosine derivatives function differently from cytosine because of their higher pK values. Thus position N^1 would be protonated and could pair with adenine (through one hydrogen bond in normal tautomeric form and two hydrogen bonds in the rare, coo1 form) instead of guanine.

The work reported here will be discussed in relation to these chemical studies. The loss of GMP-incorporating activity is directly related to the concentration of NH_2OH with which the poly C is pretreated. Under all conditions tested, the amount of AMP incorporated by poly C increased to a maximum, and then began to decrease as the time of pretreatment of the poly C with hydroxylamine increased. To look at this from another point of view, one can consider the percentage of AMP incorporated in the polymer rather than the quantity of AMP incorporated. From the data presented, it is apparent that the percentage of AMP incorporated is not linear during the treatment period. One conclusion that could be drawn from this is that a species is produced in the reaction between cytosine and hydroxylamine which causes the base pairing error, and then a second chemical species is produced which is noncomplementary to any of the normal bases. However, there are other possibilities. One of these is the removal of the altered cytosine by glycosidic bond fission, and the other is the depolymerization of the poly C. Depolymerization could account for the decrease in GMP incorporation, and the removal of the altered cytosine could account both for the continued decrease in GMP incorporation and for the decrease in the percentage of AMP incorporation in the later stages of the reaction between hydroxylamine and cytosine.

It is of interest that the absolute amount (in contrast to the percentage) of AMP incorporated was highest at low concentrations of NH_2OH. For example, at 0.2 M the peak incorporation was 400 &mu;moles of AMP; at 0.5 M, 320 &mu;moles; and at 1 M, 60 to 70 &mu;moles.

The observation that the greatest amount of AMP incorporation occurs following treatment of poly C with low concentrations of hydroxylamine can be interpreted to mean that at low concentrations of hydroxylamine the first lesion remains longer, and the AMP has a chance to be incorporated, while at higher concentrations, the second (inactivating) lesion appears so rapidly that only small amounts of AMP are incorporated.

If the previous theories (10, 12) are correct, the restoration of the double bond to the cytosine moiety should increase the GMP incorporation and decrease the AMP. Thus, if the second lesion was Compound b, acid treatment should give Compound c. However, when poly C was treated with either 0.5 M or 0.2 M NH_2OH for 0 to 6 hours, treated with acid, and then tested in the RNA polymerase system, there was no significant restoration of GMP incorporation. In fact, the level of GMP incorporation (acid-treated samples) tended to be lower than for the non-acid-treated samples. This could be accounted for on the basis of both glycosidic bond fission and deamination. Brown and Phillips (10) have reported that there was no glycosidic bond fission or deamination under these conditions, and that they did obtain some restoration of GMP incorporation by acetic acid treatment of the NH_2OH-treated polymer.

Thus, much of the evidence suggests a two-stage reaction, the first resulting in AMP incorporation and the second producing a moiety which will not pair with any of the normal bases. The evidence for these conclusions is (a) that the decrease in the quantity of AMP incorporated is not linear, (b) that AMP incorporation is greatest at low concentrations of NH_2OH, (c) that the percentage AMP incorporation is not linear, and (d) that the percentage of ApG residues decreases markedly after 6 hours. However, two other factors, glycosidic bond fission and depolymerization, cannot be ruled out completely as causes of the inactivation. Kostka and Baldwin (11) found that these were involved in the inactivation of DNA polymerase systems after treatment with nitrous acid.

It is concluded that the inactivation of poly C treated with hydroxylamine may be due to (a) the production of a nonpairable moiety from the reaction with cytosine, (b) a depolymerization which produces oligonucleotides that cannot incorporate guanine, (c) glycosidic bond fission, or (d) a combination of these. As to the species causing the replication error, it is difficult to say whether either of the known Compounds a or b in Fig. 9 represents a cytosine derivative capable of abnormal base pairing. In its normal tautomeric state, Compound a would be able to provide only one bond to adenine, and only in its rare form would it be possible to form two hydrogen bonds to adenine. The pairing to Compound b would be more difficult because the compound would have to be in its rare form to provide even one hydrogen bond with adenine. However, the principal difficulty with both of these theories is that if only one hydrogen bond were formed, it is doubtful that any base pairing would occur. Thus, both Compound a and Compound b are structurally inadequate from this standpoint. There is still the possibility that a uracil-type derivative is formed which is causing the replicative error. This was suggested by Schuster (5) and was based on both chemical and theoretical evidence. These uracil-type derivatives would arise because of the deamination of the initial addition product similar to the deamination of dihydrocytidine to dihydrodridine (16).

However, Brown and Schell (8) and Brown and Phillips (10) have rejected the presence of uracil-type derivatives on the basis that they could find little uracil in the hydroxylamine-treated samples and that the formation of Compound b from uracil-type derivatives necessitated the assumption of a rearrangement for which no analogy exists. But caution must be used in attempting to correlate chemical data with a biological phenomenon. Brown and Phillips (10), in their treatment of poly C, used 2.5 x NH_2OH (at 25°) and reported a lag in the production of Compound b under these conditions. They further reported that they could find none of Compound b (which they postulated to be the inactivating species) when they treated poly C with 1.5 M NH_2OH at 20° for 5 hours. On the basis of effect of concentration of NH_2OH on the template properties of poly C reported here, these conditions are rigorous, and their results puzzling if their original interpretation is correct. Treatment of poly C with 1 M NH_2OH and 20° for 5 hours results in a 75% reduction in...
poly C template activity as measured by GMP incorporation as reported here. In the presence of ATP and GTP this inactivation is slightly less than 75%. In the presence of 1.5 M NH₄OH the inactivation would be even greater than 75%. Thus, it is difficult to understand that if Compound b is the noncomplementary species, it cannot be detected when there is 80 to 90% inactivation of the poly C as measured enzymatically.

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