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Effects of the Carcinogen *N*-Acetoxy-2-fluorenylacetamide on the Template Properties of Deoxyribonucleic Acid

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SUMMARY

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Treatment of DNA *in vitro* with the carcinogen *N*-acetoxy-2-fluorenylacetamide (*N*-acetoxy-2-FAA) drastically reduced the capacity of the DNA to serve as a template for RNA synthesis. Significant inactivation of template activity occurred within seconds after mixing the carcinogen with DNA. A given quantity of treated DNA could bind 10 times more RNA polymerase than the same quantity of control DNA. RNA synthesis directed by treated DNA ceased after 5 min of incubation, while RNA synthesis directed by control DNA continued for over 1 hr. Treated DNA was as effective as control DNA in supporting the exchange of [^{32}P]pyrophosphate into nucleoside triphosphates in the presence of RNA polymerase. These findings indicated that *N*-acetoxy-2-FAA inhibited RNA synthesis by preventing chain elongation, and that chain initiation was unaffected. Denaturation by heat or alkali decreased the template capacity of control DNA but increased the template capacity of treated DNA. In addition, treated DNA was more effective than control DNA in supporting the homopolymerization of adenylic acid by RNA polymerase. It therefore appeared likely that *N*-acetoxy-2-FAA produced regions of partial denaturation of DNA. Comparison of the effects of a series of *N*-acetoxyarylacetamides revealed that abolition of DNA template activity only occurred when the aryl moiety was greater than one ring in size and when the nitrogen atom was located *para* to the aromatic system.

INTRODUCTION

The interaction of chemical carcinogens with cellular constituents has long been regarded as essential to the carcinogenic process (1, 2). Metabolites of the potent hepatocarcinogen, *N*-hydroxy-2-fluorenylacetamide, are bound to liver DNA upon administration of the carcinogen to susceptible species (3–9). The functional significance of this binding and its role in carcinogenesis are unknown.

N-Hydroxy-2-FAA[2] does not react to any significant extent with nucleic acids *in vitro*, but its acetate ester, *N*-acetoxy-2-FAA,

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[2] The abbreviations used are: *N*-hydroxy-2-FAA, *N*-hydroxy-2-fluorenylacetamide; *N*-acetoxy-2-FAA, *N*-acetoxy-2-fluorenylacetamide.

reacts nonenzymatically with DNA under physiological conditions (10). The major product of this reaction is DNA-bound 8-(*N*-2-fluorenylacetamido)guanine (11). Treatment of DNA with *N*-acetoxy-2-FAA has profound effects on the biological properties of the DNA. For example, Maher *et al.* (12) showed that *N*-acetoxy-2-FAA caused point mutations, and Troll *et al.* (13) found that treatment with *N*-acetoxy-2-FAA drastically reduced the capacity of DNA to support RNA synthesis in the presence of RNA polymerase.

During a study of this inactivation of DNA template activity it was observed that *N*-acetoxy-2-FAA did not merely inhibit RNA synthesis but also altered the characteristics of the RNA polymerase reaction. These observations suggested that, in addition to producing a quantitative inhibition of RNA synthesis, *N*-acetoxy-2-FAA might have subtler effects on the reaction, which could affect the types of RNA species synthesized. The experiments described in this report deal with the template properties of *N*-acetoxy-2-FAA-treated DNA in the RNA polymerase reaction and with the structural requirements for the abolition of DNA template activity.

EXPERIMENTAL PROCEDURE

Preparation of DNA. Two methods were used to prepare DNA from the livers of normal male Holtzman rats. The first was the method of Savitsky and Stand (14). The final DNA preparation was dissolved in 0.015 M NaCl plus 0.0015 M sodium citrate (pH 7.0), treated with RNase (40 μg/ml) for 1 hr at 37°, and then incubated with Pronase (100 μg/ml) for 1 hr at 37°. The mixture was then extracted twice with phenol in the presence of 1% sodium dodecyl sulfate, and the DNA was precipitated with ethanol, dissolved in 0.015 M NaCl plus 0.015 M sodium citrate (pH 7.0), and dialyzed against this solution. The second method of preparation was that described by Irving and Veazey (15). Following RNase treatment the mixture was treated with Pronase and extracted with phenol as described above, and subsequently precipitated with 2-propanol (15). No significant differences were noted between DNA preparations of the two types. The DNA solutions were stored in liquid N_2.

Preparation of RNA polymerase. RNA polymerase from *E. coli* B was purified by the method of Burgess (16). The final purification was achieved by glycerol gradient centrifugation. The enzyme incorporated 680 nmoles of AMP per milligram of protein in the assay system described below, using 200 μg/ml of calf thymus DNA as template.

Preparation of compounds. *N*-Acetoxy-2-FAA, m.p. 110–112° (17), and *N*-hydroxy-2-FAA, m.p. 150.5–151.5° (18), were prepared by published methods. *N*-Hydroxy-2-FAA was further purified by conversion to its copper chelate (19). The chelate was decomposed with hydrogen sulfide, and the product was recrystallized from aqueous ethanol, m.p. 149–151°.

The remaining hydroxamic acid esters were gifts from Yul Yost, to whom I am deeply indebted. *N*-Acetoxy-1-fluorenylacetamide, m.p. 77–79° (20), *N*-acetoxy-3-fluorenylacetamide, m.p. 104–105° (21), *N*-acetoxy-4-biphenylylacetamide, m.p. 118° (22), and *N*-acetoxyphenylacetamide, m.p. 36–38° (23), were prepared by published procedures. The new compounds, which were prepared by acetylation of the respective hydroxamic acids in pyridine with acetic anhydride (20), were *N*-acetoxy-4-fluorenylacetamide, m.p. 108°, λ_{max}^{EtOH} 261 (ε 15,200), 296 (ε 8600), and 303 (ε 10,000) nm, ν_{max}^{KBr} 1793 (O—C═O) and 1693 (N—C═O) cm^{-1}; *N*-acetoxy-2-biphenylylacetamide, m.p. 101–102°, λ_{max}^{EtOH} 232 (ε 32,200) nm, ν_{max}^{KBr} 1790 (O—C═O) and 1685 (N—C═O) cm^{-1}; and *N*-acetoxy-3-biphenylylacetamide, m.p. 83°, λ_{max}^{EtOH} 242 (ε 26,000) nm, ν_{max}^{KBr} 1795 (O—C═O) and 1685 (N—C═O) cm^{-1}. All the new compounds had the correct elementary compositions.

Reaction of DNA with N-acetoxy-2-FAA. Incubation mixtures contained 50 mM Tris-Cl (pH 7.9), 15 mM NaCl, 1.5 mM sodium citrate, 30% (v/v) ethanol, 5% (v/v) dimethylsulfoxide, 100 μg/ml of DNA, and *N*-acetoxy-2-FAA as indicated. The volume of the mixture was typically 1 ml, and incubation was carried out for 1 hr at 23°. After incubation the DNA was precipitated by the addition of 100 μl of 5 M NaCl and 2 ml of 95% ethanol. The precipitate was washed

four times with 3 ml of 95% ethanol and dissolved in 200 μl of 15 mM NaCl plus 1.5 mM sodium citrate (pH 7.0). The solution was extracted four times with 1 ml of chloroform and twice with 1 ml of water-saturated ether, and finally dialyzed overnight against 15 mM NaCl plus 1.5 mM sodium citrate (pH 7.0). Control DNA was treated in exactly the same manner, but without the addition of *N*-acetoxy-2-FAA. This control DNA was indistinguishable from untreated rat liver DNA in its thermal melting profile and in its template capacity for RNA synthesis.

RNA polymerase assay. The reaction mixture (75 μl, final volume) contained 40 mM Tris-Cl (pH 7.9), 4 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM dithiothreitol, 0.5 mg/ml of bovine serum albumin, 0.4 mM GTP, 0.4 mM CTP, 0.4 mM UTP, 0.4 mM [^{14}C]ATP (5 mCi/mmole), and DNA and enzyme as indicated. Incubation was performed for 10 min at 37°, and acid-insoluble radioactivity was determined by the method of Bollum (24).

RESULTS

In agreement with the findings of Troll *et al.* (13), the incubation of native rat liver DNA with *N*-acetoxy-2-FAA substantially reduced the ability of the DNA to serve as a template for RNA synthesis. Figure 1 shows this inhibition of template activity as a function of the concentration of *N*-acetoxy-2-FAA with which the DNA was incubated. In these experiments the DNA concentration was kept constant at 100 μg/ml and the *N*-acetoxy-2-FAA concentration was varied through the range shown on the abscissa. Concentrations of *N*-acetoxy-2-FAA as low as 10 μM inhibited DNA template activity significantly, while concentrations of 1 mM and above almost completely abolished RNA synthesis.

Troll *et al.* (25) have also reported a substantial decrease in DNA template activity following incubation with *N*-hydroxy-2-FAA. Under the present conditions *N*-hydroxy-2-FAA had no effect on template activity at concentrations below 1 mM and produced a maximal inhibition of 18% at higher concentrations (Fig. 1). The *N*-hydroxy-2-FAA used in these experiments was purified by conversion to its copper chelate.

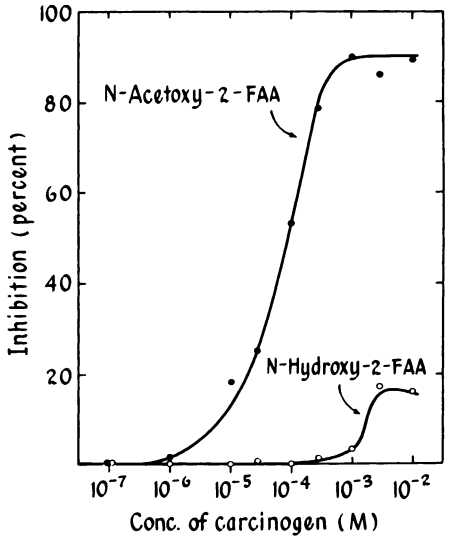


FIG. 1. *Inhibition of RNA synthesis by treatment of DNA with N-acetoxy-2-FAA or N-hydroxy-2-FAA*

DNA was incubated with the indicated concentration of *N*-acetoxy-2-FAA or *N*-hydroxy-2-FAA for 60 min at 23°. The remaining components of the reaction mixtures were as described under EXPERIMENTAL PROCEDURE, except that the concentration of dimethylsulfoxide was 10% (v/v). RNA polymerase assays contained 10 μg of enzyme and 4 μg of DNA in a volume of 75 μl. Under these conditions 2400 pmoles of AMP were incorporated into RNA in 10 min at 37° in the presence of untreated rat liver DNA.

When apparently homogeneous *N*-hydroxy-2-FAA which had not been purified by chelate formation was used, a maximal inhibition of 45% was observed. This is comparable to the 50% inhibition reported by Troll *et al.* (25), who used a preparation of *N*-hydroxy-2-FAA which had not been purified.

Figure 2 shows the time course of the inactivation of DNA template activity by *N*-acetoxy-2-FAA. DNA incubated with *N*-acetoxy-2-FAA for 30 sec at 23° exhibited a significantly reduced capacity to support RNA synthesis, and 50% inhibition was achieved within 3 min. The inactivation proceeded at a diminishing rate throughout the incubation.

The rat liver RNA polymerases are inactivated following a single injection of *N*-hydroxy-2-FAA (26). Because of this inactiva-

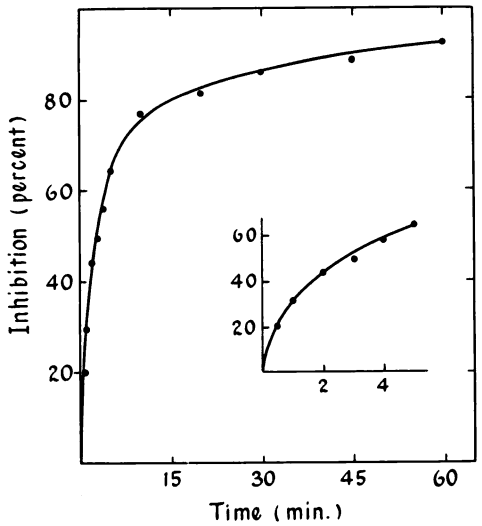


FIG. 2. *Time course of inactivation of DNA template activity by N-acetoxy-2-FAA*

DNA was incubated at 23° with 1 mM *N*-acetoxy-2-FAA for the indicated time periods. The composition of the reaction mixtures was as described under EXPERIMENTAL PROCEDURE. RNA polymerase assays contained 10 μg of enzyme and 4 μg of DNA in a volume of 75 μl.

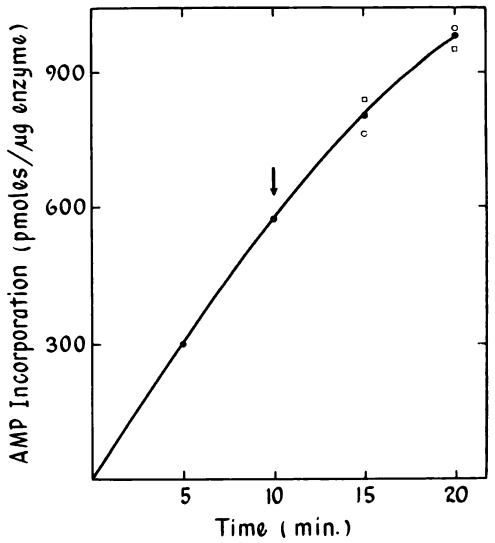


FIG. 3. *Effect of N-acetoxy-2-FAA-treated DNA on transcription of control DNA*

DNA was incubated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23° as described under EXPERIMENTAL PROCEDURE. The reaction mixture for RNA synthesis (500 μl, final volume) contained 100 μg of control liver DNA and 20 μg of RNA polymerase. After incubation for 10 min at 37°, three 100-μl aliquots, containing 20 μg of DNA, were removed. These aliquots were mixed with 20 μl of buffer (●——●), 20 μl of buffer containing 20 μg of normal liver DNA (○——○), or 20 μl of buffer containing 20 μg of *N*-acetoxy-2-FAA-treated DNA (□——□). The buffer was 15 mM NaCl plus 1.5 mM sodium citrate (pH 7.0). After mixing, the incubation was continued at 37°. Samples (50 μl) were taken at 5-min intervals for the determination of acid-insoluble radioactivity.

tion *in vivo*, it was relevant to ask whether the observed inhibition of RNA synthesis in the present experiments *in vitro* might be due to inactivation of the enzyme by traces of *N*-acetoxy-2-FAA which were carried along with the DNA through the washing procedure. To test this possibility, an RNA polymerase reaction mixture was set up which contained sufficient control liver DNA to saturate the enzyme. The mixture was incubated for 10 min at 37°. Under these conditions all the enzyme present is bound virtually irreversibly to the DNA (27). After 10 min of incubation the reaction mixture was divided into three aliquots. To one of these was added buffer, to the second additional control DNA, and to the third DNA treated with *N*-acetoxy-2-FAA. If the inhibition of RNA synthesis were due to enzyme inactivation, one would expect the addition of treated DNA to depress the rate of RNA synthesis from control DNA. The results shown in Fig. 3 indicate that this was not the case; addition of treated DNA had no effect on the rate of transcription of control DNA. Therefore the inhibition of RNA synthesis by *N*-acetoxy-2-FAA probably reflects inactivation of the template rather than of the enzyme.

Besides being much less able to support RNA synthesis, the *N*-acetoxy-2-FAA-treated DNA differed from the control DNA in several of its properties. Figure 4 shows the initial rates of RNA synthesis primed by control and treated DNA as a function of the amount of DNA added to the incubation mixture. A constant amount (10 μg) of RNA polymerase was used in these incubations. It is apparent that the treated DNA saturated the enzyme at a much lower concentration than the control

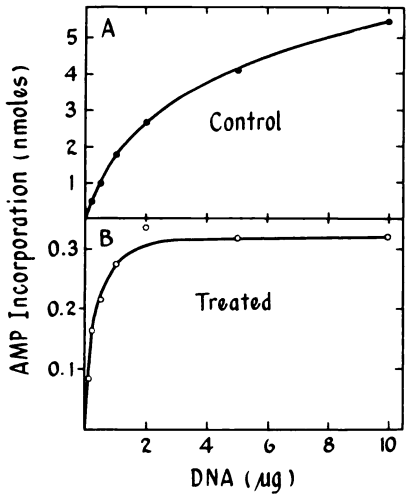


FIG. 4. *Rates of RNA synthesis as a function of DNA concentration*

DNA was treated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23°. RNA polymerase assay mixtures (75 μl) contained 10 μg of enzyme and the indicated amount of DNA. A. Control DNA. B. *N*-Acetoxy-2-FAA-treated DNA.

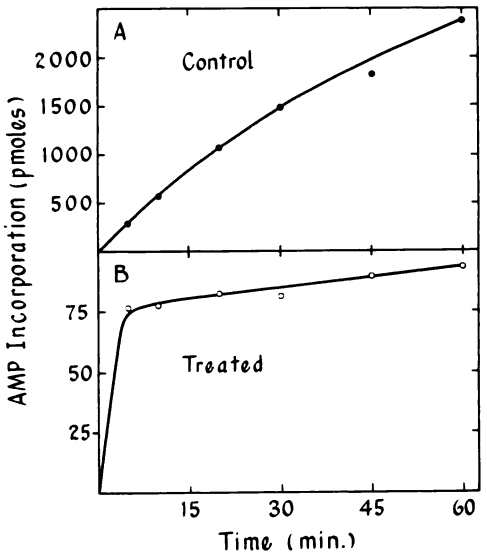


FIG. 5. *Time course of RNA synthesis in the presence of control or N-acetoxy-2-FAA-treated DNA*

DNA was incubated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23°. RNA polymerase assay mixtures contained 2 μg of enzyme and 10 μg of DNA in a volume of 50 μl. The tubes were incubated at 37° for the indicated times. A. Control DNA. B. *N*-Acetoxy-2-FAA-treated DNA.

DNA. Approximately 0.2 μg of treated DNA half-saturated 10 μg of RNA polymerase, while 2 μg of control DNA were required for half-saturation of the same amount of enzyme.

The time courses of RNA synthesis directed by control DNA and by DNA treated with *N*-acetoxy-2-FAA are given in Fig. 5. In these experiments sufficient DNA was added to saturate the amount of enzyme present. Under these conditions the initiation of RNA chains is negligible after the first few minutes of the reaction (28). When control DNA was used as the template (Fig. 5A), the incorporation of AMP proceeded linearly for approximately 20 min and continued at a gradually diminishing rate throughout the period of incubation (1 hr). In contrast, when treated DNA was used as the template (Fig. 5B), the incorporation was virtually complete by the time the first aliquot was removed after 5 min of incubation. Thus the AMP incorporation at 5 min was 82% of the incorporation at 60 min. Since termination and reinitiation of RNA chains do not occur under these conditions (28), the early cessation of RNA synthesis suggests that the treatment of DNA with *N*-acetoxy-2-FAA inhibits RNA chain elongation.

It is possible, of course, that *N*-acetoxy-2-FAA might inhibit the initiation of RNA chains as well as their elongation. As a measure of chain initiation, the capacity of control and treated DNA to support the incorporation of labeled inorganic pyrophosphate into nucleoside triphosphates was tested. Krakow and Fronk (29) have shown that pyrophosphate exchange occurs at the dinucleotide level by the successive formation and pyrophosphorolysis of the first phosphodiester bond in the RNA chain. Figure 6 shows the rate of pyrophosphate exchange catalyzed by RNA polymerase in the presence of normal and treated DNA. It is apparent that *N*-acetoxy-2-FAA had no significant effect on the capacity of DNA to support this exchange reaction. The treated DNA used in this experiment had less than 5% of the template capacity of normal DNA, as measured by the incorporation of AMP into RNA. Hence it appears

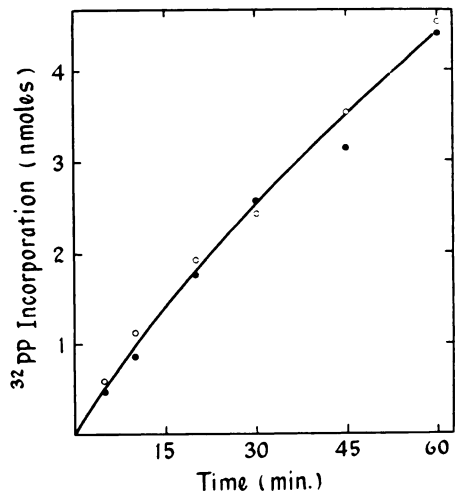


FIG. 6. *$^{32}PP_i$-nucleoside triphosphate exchange in the presence of control or treated DNA*

DNA was treated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23°. For the measurement of pyrophosphate exchange, the reaction mixtures (50 μl) contained 50 mM Tris-Cl (pH 7.9), 5 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.4 mM each ATP, GTP, CTP, and UTP, 1 mM $^{32}PP_i$, 4 μg of DNA, and 10 μg of RNA polymerase. The incubation temperature was 37°. At the indicated time intervals the reaction was stopped by the addition of 100 μl of 0.1 M EDTA, followed by 1 ml of 10 mM $Na_4P_2O_7$ (adjusted to pH 6.0 with KH_2PO_4) containing 5% (w/v) charcoal. The charcoal was collected on a glass fiber filter and washed with 10 mM $Na_4P_2O_7$ (pH 6.0). The filters were dried under an infrared lamp and counted with the use of a toluene-based scintillator solution. ●, control DNA; ○, treated DNA.

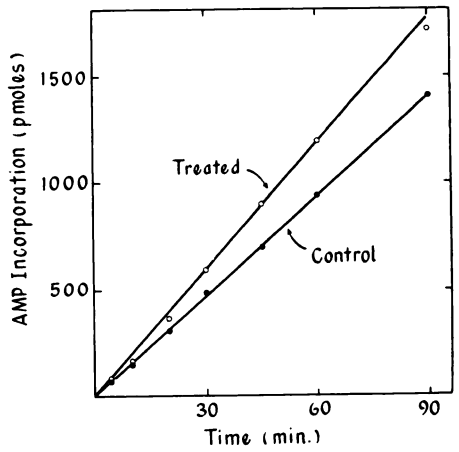


FIG. 7. *Synthesis of polyadenylic acid by RNA polymerase in the presence of control or N-acetoxy-2-FAA-treated DNA*

DNA was incubated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23°. The synthesis of polyadenylic acid was assayed in reaction mixtures (50 μl, final volume) containing the standard components of the RNA polymerase assay, except that GTP, CTP, and UTP were omitted. There were 2 μg of enzyme and 10 μg of DNA present, and the incubation temperature was 37°.

that *N*-acetoxy-2-FAA inhibits RNA synthesis primarily by preventing chain elongation, and that chain initiation is unaffected.

The major product of the reaction of *N*-acetoxy-2-FAA with the constituents of DNA is 8-(*N*-2-fluorenylacetamido)guanine (11). It was therefore of interest to determine whether regions of the treated DNA which did not contain guanine could serve as templates for the polymerization of ribonucleotides. The DNA-dependent synthesis of polyadenylic acid, which is thought to occur by the reiterative transcription of thymine clusters in DNA (30), affords a system in which the template capacities of certain non-guanine-containing DNA regions can be measured. Figure 7 gives the rates of polyadenylic acid synthesis by RNA polymerase in the presence of control and treated DNA. The treated DNA supported a somewhat greater rate of AMP polymerization than the control DNA, in sharp contrast to the decreased ability of the treated DNA to support RNA synthesis in the presence of all four nucleoside triphosphates.

The homopolymerization of AMP is thought to require single-stranded DNA as a template (30). The increased rate of polyadenylic acid formation in the presence of treated DNA raised the question whether a major effect of *N*-acetoxy-2-FAA treatment could be to denature the DNA. Troll *et al.* (25) have reported that *N*-acetoxy-2-FAA decreases the temperature of half-melting of DNA. The thermal melting profiles of normal rat liver DNA and of DNA treated with two different concentrations of *N*-acetoxy-2-FAA are shown in Fig. 8. Treatment of DNA with 10 mM *N*-acetoxy-2-FAA lowered the T_M by 7.4° and reduced the relative hyperchromicity on melting by 10%. In contrast, treatment with 1 mM *N*-acetoxy-2-FAA had no discernible effect on the melting

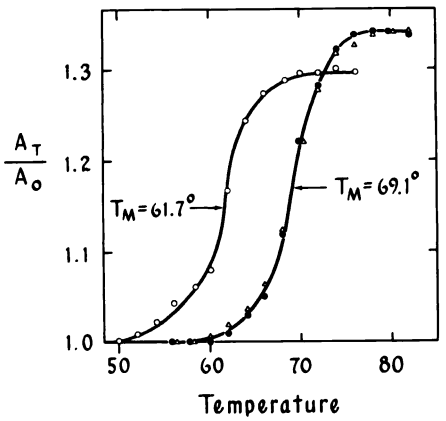


FIG. 8. *Effect of N-acetoxy-2-FAA on thermal melting profile of DNA*

DNA was incubated with *N*-acetoxy-2-FAA for 60 min at 23°. The concentration of the carcinogen was 1 mM (△——△) or 10 mM (○——○). Melting profiles were measured in 15 mM NaCl plus 1.5 mM sodium citrate (pH 7.0) after the DNA samples had been dialyzed against this buffer for 48 hr. ●——●, control DNA.

profile. Both these treated DNA preparations had less than 10% of the template activity of control DNA, as shown in Fig. 1. It therefore appears that the inhibition of template activity for RNA synthesis can take place in the absence of detectable destabilization of the double-helical structure of native DNA.

In view of the denaturation-like changes in the template properties of DNA treated with *N*-acetoxy-2-FAA, it was of interest to examine the effect of denaturation on the template capacity of the treated DNA. Denaturation was accomplished by treatment with alkali at 23° or by heating at 95° followed by rapid cooling. In agreement with previous reports (31, 32), the denaturation of control DNA decreased its efficacy as a template for RNA synthesis (Table 1). In contrast, denaturation of treated DNA by alkali or by heat increased its template capacity significantly. Native *N*-acetoxy-2-FAA-treated DNA had only 12% of the template capacity of native control DNA, while alkali-denatured treated DNA had 68% of the template capacity of alkali-denatured control DNA and heat-denatured treated DNA had 57% of the template capacity of heat-denatured control DNA.

TABLE 1

Effect of denaturation on template capacity of normal and N-acetoxy-2-FAA-treated DNA

DNA was treated with 1 mM *N*-acetoxy-2-FAA for 60 min at 23°. DNA was denatured by treatment with 0.2 N NaOH for 10 min at 23° or by heating for 10 min at 95° followed by rapid cooling. RNA polymerase reaction mixtures contained 10 μg of enzyme and 10 μg of DNA in a final volume of 75 μl. Similar results were obtained with three different preparations of treated DNA.

Condition of DNA	AMP incorporation	
	Control	*N*-Acetoxy-2-FAA
	pmoles	
Native	5067	630
Alkali-denatured	1440	982
Heat-denatured	1913	1093

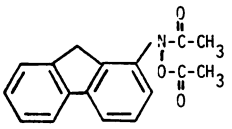
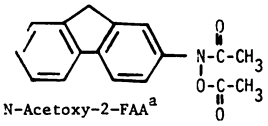
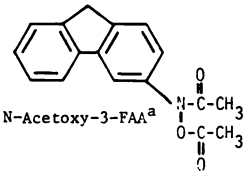
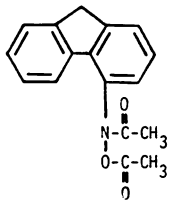
The carcinogenicity of arylhydroxamic acids depends upon several structural features of the molecule. Among these are the size of the aryl moiety and the position of the nitrogen with respect to the aromatic system (33). The availability of a general procedure for the synthesis of hindered arylhydroxamic acids and their acetate esters (34) made it possible to test the effects of these structural features on the ability of esters to inactivate the template activity of DNA *in vitro*. Table 2 shows the effects of the acetate esters of fluorenylacetohydroxamic acids substituted at various positions in the fluorene system on the template capacity of DNA. Only *N*-acetoxy-2-fluorenylacetamide inhibited RNA synthesis significantly. Prolonged incubation with concentrations as high as 10 mM *N*-acetoxy-1-, 3-, or 4-fluorenylacetamide had no effect on DNA template activity.

A similar situation was observed with the acetate esters of the various biphenylylacetohydroxamic acids shown in Table 3. In this case the only compound which produced significant inhibition of RNA synthesis was *N*-acetoxy-4-biphenylylacetamide. The inhibition observed, however, was significantly less than that produced under the same conditions by *N*-acetoxy-2-FAA. Within the limits of sensitivity of the assay system, DNA treated with *N*-acetoxy-2-biphenylyl-

TABLE 2

Effects of acetate esters of fluorenylacetohydroxamic acids on template activity of DNA

DNA was incubated with each ester as described under EXPERIMENTAL PROCEDURE for the incubation of DNA with *N*-acetoxy-2-fluorenylacetamide. The concentration of the ester was 1 mM, and the incubation time was 60 min at 23°. RNA polymerase assay mixtures contained 10 μg of enzyme and 4 μg of DNA in a volume of 75 μl. Each value represents the mean ± standard deviation for three DNA preparations.

Treatment of DNA	RNA synthesis	
	AMP incorporation	Inhibition
	pmoles	%
Control	2388 ± 109	—
 N-Acetoxy-1-FAA[a]	2507 ± 97	0
 N-Acetoxy-2-FAA[a]	178 ± 31	93
 N-Acetoxy-3-FAA[a]	2421 ± 79	0
 N-Acetoxy-4-FAA[a]	2291 ± 113	4

[a] -FAA, -fluorenylacetamide.

acetamide or *N*-acetoxy-3-biphenylylacetamide was indistinguishable from untreated DNA. *N*-Acetoxyphenylacetamide, in which the aromatic system is a phenyl group, was also without detectable effect on DNA template capacity.

DISCUSSION

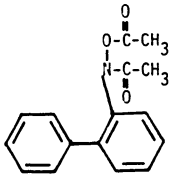
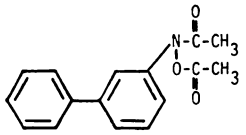
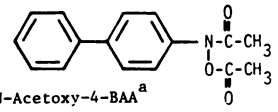
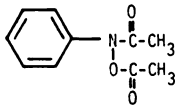
N-Acetoxy-2-FAA is capable of almost completely eliminating the template activity of DNA, as noted earlier by Troll *et al.* (13). In the present system 50% inhibition was achieved at a concentration of 0.1 mM *N*-acetoxy-2-FAA (Fig. 1). Since the concentration of DNA in these reactions was 100 μg/ml, this corresponds to a ratio of approximately 0.3 mole of *N*-acetoxy-2-FAA per mole of DNA nucleotide in the incubation mixture.

No direct measurements were made of the fraction of DNA guanine residues modified by *N*-acetoxy-2-FAA in the present experiments. A maximum value for the extent of substitution can be obtained by comparing

TABLE 3

Effects of acetate esters of biphenylylacetohydroxamic acids and of N-hydroxyphenylacetamide on template activity of DNA

The incubation of DNA with the various esters and the RNA polymerase assays were carried out as described in the legend to Table 2.

Treatment of DNA	RNA synthesis	
	AMP incorporation	Inhibition
	pmoles	%
Control	2388 ± 109	—
 N-Acetoxy-2-BAA[a]	1996 ± 52	12
 N-Acetoxy-3-BAA[a]	2245 ± 96	6
 N-Acetoxy-4-BAA[a]	1504 ± 80	37
 N-Acetoxyphenylacetamide	2402 ± 66	0

[a] -BAA, -biphenylylacetamide.

the thermal melting profiles of Fig. 8 with those obtained by Fuchs and Daune (35) and by Kapuler and Michelson (36). These authors found that modification of approximately 8% of the total guanine residues of *Micrococcus lysodeikticus* DNA lowered the T_M of the DNA by 2°, a difference which would have been detected by the techniques used in the present work. Treatment of rat liver DNA with 1 mM *N*-acetoxy-2-FAA did not detectably alter the T_M of the DNA, but reduced its template capacity by more than 90%. It may therefore be concluded that the modification of less than 8% of the guanine residues in DNA by *N*-acetoxy-2-FAA results in almost complete inhibition of RNA synthesis.

At a concentration of 1 mM, *N*-acetoxy-2-FAA inactivated DNA rapidly, with 3 min of incubation being required to achieve 50% inhibition. In the later part of the incubation the rate of inactivation was drastically reduced, which appears inconsistent with the report by Maher *et al.* (12) that the half-life of *N*-acetoxy-2-FAA in 32% ethanol at pH 7.5 and room temperature was approximately 2 hr. These authors suggested, how-

ever, that the half-life of the ester might be shorter in the presence of DNA.

DNA treated with *N*-acetoxy-2-FAA saturated a given quantity of RNA polymerase at a 10-fold lower concentration than control DNA. The values for half-saturation calculated from Fig. 4 should not be regarded as Michaelis constants, since the association of RNA polymerase with DNA is known to be irreversible in the presence of RNA synthesis (27). In keeping with this reasoning, the DNA concentration required for half-maximal reaction velocity depends on the quantity of enzyme present (37). Hence the saturation curves in Fig. 4 can best be interpreted as reflecting the number of polymerase binding sites on the DNA (37, 38). A given weight of treated DNA is therefore capable of binding more RNA polymerase than the same weight of control DNA. Denatured DNA also possesses more polymerase binding sites per unit weight than native DNA, and so does DNA damaged by γ-irradiation (39, 40), ultraviolet irradiation (41), deoxyribonuclease (40), and hydroxylamine (42).

The increased efficacy of the treated DNA in supporting polyadenylic acid synthesis suggested that the treatment produced regions of denaturation of the DNA. It is apparent (Fig. 8 and ref. 25) that high concentrations of *N*-acetoxy-2-FAA produced a gross destabilization of DNA toward thermal denaturation. The treated DNA used in most of the present studies, however, had a thermal melting profile which was indistinguishable from that of untreated DNA (Fig. 8). Since this treated DNA had less than 10% of the template capacity of normal DNA, it may be concluded that the decrease in T_M upon treatment with *N*-acetoxy-2-FAA is not directly related to the decrease in template capacity. Some localized denaturation is presumably produced by 1 mM *N*-acetoxy-2-FAA, in view of the increase in template activity for homopolymer synthesis. The denatured regions must be relatively short, however, since the melting profile is unaffected.

The increase in the template capacity of *N*-acetoxy-2-FAA-treated DNA upon denaturation by alkali or by heat was somewhat surprising. It is conceivable that some fluorenyl adducts were cleaved from the DNA by 0.2 N NaOH. Indeed, Kriek (7) and Irving *et al.* (8) have found that the DNA adducts formed after administration of *N*-hydroxy-2-FAA *in vivo* are alkali-labile. The adducts *in vivo*, however, are deacetylated (7, 8, 43), while the adducts formed *in vitro* from *N*-acetoxy-2-FAA retain the acetyl group (11). The increased template capacity following heat denaturation is less likely to result from cleavage of adducts, since the product of the reaction of *N*-acetoxy-2-FAA with DNA is unaffected by prolonged incubation at neutral pH (25).

The paradoxical effects of denaturation on the template capacity of treated DNA might be due in part to interstrand cross-links produced by the carcinogen. Such cross-links have been shown to occur (35), and if they were present in sufficiently small number, they might not affect the thermal melting profile. These cross-links would prevent strand separation upon denaturation of the treated DNA, and hence would tend to prevent the decrease in template activity seen upon denaturation of normal DNA.

Comparison of the effects of the acetate esters of various arylacetohydroxamic acids revealed that only the *para*-substituted compounds (*N*-acetoxy-2-FAA and *N*-acetoxy-4-biphenylylacetamide) produced detectable inhibition of DNA template activity. Both these compounds are highly carcinogenic (44). *N*-Hydroxy-3-fluorenylacetamide is also highly carcinogenic (33), but its acetate ester, *N*-acetoxy-3-fluorenylacetamide, had no effect on template activity. This indicates either that *N*-acetoxy-3-fluorenylacetamide did not react with DNA or that, if it did react, the resulting adducts did not inhibit RNA synthesis. In support of the first alternative is the failure of *N*-acetoxy-3-fluorenylacetamide to react with methionine at neutral pH (21). If *N*-acetoxy-3-fluorenylacetamide is unreactive under physiological conditions, then it is likely that carcinogenesis by *N*-hydroxy-3-fluorenylacetamide requires metabolic activation of the hydroxamic acid by some process other than esterification.

The inhibition of RNA synthesis by hy-

droxamic acid esters was also dependent on the size and rigidity of the aryl moiety. Thus replacement of the planar fluorene group by the more flexible biphenyl group reduced the degree of inhibition, while replacement by the phenyl group completely eliminated the inhibition. These results correlated well with the carcinogenicities of the corresponding arylhydroxamic acids (33).

It was shown previously that the administration of a single dose of *N*-hydroxy-2-FAA to male rats causes a profound inhibition of liver RNA synthesis (26). This inhibition *in vivo* is due to decreased activity of the liver RNA polymerases rather than to decreased template capacity of liver DNA. The experiments described in the present report have shown that *N*-acetoxy-2-FAA can also produce profound alterations in the template properties of DNA. Thus *N*-hydroxy-2-FAA and its esters are capable of inhibiting RNA synthesis both at the level of the enzymes and at the level of the DNA template. The production of hepatomas by *N*-hydroxy-2-FAA requires prolonged or repeated administration of the compound. It remains to be seen whether either or both of these mechanisms for altering cellular RNA synthesis are operative under such conditions of chronic administration.

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