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Inhibition of RNA Synthesis by Derivatives of the Carcinogen 2-Acetylaminofluorene[1] (40524)

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Acute administration of the hepatocarcinogen *N*-hydroxy-*N*-2-acetylaminofluorene (N-OH AAF) to rats or mice rapidly inhibits hepatic RNA synthesis. (1–3). It is thought that N-OH AAF is converted to an ester (possibly a sulfate ester), derivatives of which bind covalently to cellular macromolecules and cause metabolic alterations (4, 5). Previous work by Yu and Grunberger (6) has suggested that inhibition of the enzyme RNA polymerase II may be important in the reduction of nucleoplasmic RNA synthesis whereas inhibition of template activity may be the important factor leading to reduced rRNA synthesis. To investigate this phenomenon further we have studied the sensitivities of RNA polymerases I and II as well as DNA template to direct inactivation by *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-acetoxy AAF), an active ester of N-OH AAF which has been used as a model for *in vivo* binding of AAF to cellular macromolecules (7, 8).

Materials and methods. AAF and N-OH AAF were obtained from the Aldrich Chemical Co., Milwaukee, WI. *N*-acetoxy AAF and *N*-acetoxy 9-[^{14}C]AAF were synthesized by the technique of Irving and Veazey (9). 9-[^{14}C]N-OH AAF was obtained from ICN, Irvine, CA. The absence of detectable impurities in these carcinogens was determined by a three solvent thin layer chromatography system (10). Melting points of AAF, N-OH AAF, and *N*-acetoxy AAF were 192°, 147°, and 108° respectively and analysis of infrared spectra confirmed their identities.

Male Sprague-Dawley rats (150–180 g) were maintained on Purina Lab Chow and were fasted for 12–16 hr before experiments.

RNA polymerase. Enzyme was purified from liver nuclei and fractionated into forms I, II and III by the method of Roeder and Rutter (11). RNA polymerase was assayed as described (12) using 100 μM [^{3}H]UTP as labeled precursor, 1 mM $MnCl_2$, and salt concentrations appropriate for the particular activity being measured (See Table Legends).

Treatment of RNA polymerase with N-acetoxy AAF. Enzyme fractions were incubated at 25° for 1 hr in 0.01 M Tris–HCl, pH 7.4 and 13% dimethyl sulfoxide (DMSO) in the presence or absence of *N*-acetoxy AAF. Proteins were then precipitated with $(NH_4)_2SO_4$ (40% saturation), washed repeatedly by resuspension in 50% saturated $(NH_4)_2SO_4$ in 0.05 M Tris–HCl (pH 7.7) -25% glycerol-5 mM $MgCl_2$-1 mM EDTA – 0.01 M dithiothreitol (TGMED) and resuspended in TGMED. Incubation of the enzyme with DMSO followed by precipitation and washing in this manner had no effect on subsequent enzyme activity. Where binding of labeled *N*-acetoxy AAF to the enzyme was to be determined, the above incubation was followed by four extractions with H_2O-saturated ether. The protein was then precipitated with 7% perchloric acid, and the resuspended pellets treated with pancreatic RNase (20 μg/ml) in 2.5 mM Tris–HCl (pH 7.5) for 2 hr at 37°. After reprecipitation with perchloric acid, radioactivity and protein concentration (13) were determined on the resuspended samples.

Treatment of DNA with N-acetoxy AAF. Calf thymus DNA (1–5 mg, 200 μM) was incubated in 0.01 M Tris–HCl (pH 7.4) for 1 hr at 25° with unlabeled or [^{14}C]labeled (0.085 mCi/mmol) *N*-acetoxy AAF. After four extractions with H_2O-saturated ether and ethanol precipitation the DNA pellets were washed with ethanol and dissolved in 0.05 M Tris–HCl (pH 7.7), 0.003 M $MgCl_2$. DNA not exposed to *N*-acetoxy AAF but processed in the same way was as effective a template for RNA polymerase as untreated DNA. To determine binding of [^{14}C]*N*-acetoxy AAF to DNA the above washing pro-

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cedure was followed by hydrolyzing the DNA overnight with pancreatic DNase (50 μg/ml) followed by precipitation with perchloric acid and determination of counts and DNA concentration (14) on the supernatants.

Administration of N-OH AAF or [^{14}C]N-OH AAF to rats. Animals were injected intraperitoneally with N-OH AAF (30 mg/kg) in DMSO or with solvent alone and sacrificed 60 min later. To determine binding of AAF derivatives to cellular components *in vivo*, enzyme or DNA was purified from rat liver and then extracted as described for the *in vitro* binding experiments.

Results. Inhibition of RNA synthesis in isolated nuclei. A single intraperitoneal injection of N-OH AAF produces a dramatic but reversible inhibition of rat liver RNA synthesis as measured in isolated nuclei (Table I, Experiment 1). To determine if this effect could be due to direct inactivation of enzyme or template by reaction with an AAF derivative, normal liver nuclei were incubated *in vitro* with *N*-acetoxy AAF, a possible ultimate carcinogen of AAF, and the subsequent ability of the nuclei to carry out RNA synthesis was determined (Table I, Experiment 2). Preincubation of the nuclei with 10^{-3} *M N*-acetoxy AAF results in almost total inhibition of nuclear RNA synthesis, supporting a direct inactivation model. When *N*-acetoxy AAF-treated nuclei were assayed using an exogenous template poly d(AT), inhibition of RNA synthesis was again observed. Since *N*-acetoxy AAF reacts to a negligible extent with poly d(AT) under these conditions (15, 16), this result suggests that an AAF derivative may be capable of directly inactivating one or more of the classes of RNA polymerase.

Inactivation of template or enzyme by N-acetoxy AAF. Table II, Experiment 1 shows that when DNA was arylamidated with *N*-acetoxy AAF and then used as a template for *in vitro* RNA synthesis, transcription is less than that obtained using untreated DNA. To obtain substantial inhibition of transcription a high concentration of *N*-acetoxy AAF (10^{-3} *M*) is required. Diminished template activity is apparent whether crude RNA polymerase, polymerase I or polymerase II is used in the assay.

Table II, Experiment 2, shows that interaction of *N*-acetoxy AAF with RNA polymerase directly inactivates the enzymes. Inhibition of both polymerase I and polymerase II was obtained by preincubating the enzyme with 10^{-3} *M N*-acetoxy AAF. RNA polymerase II, the more sensitive of the enzymes to inhibition, is significantly inhibited by a concentration of N-acetoxy AAF as low as 10^{-4} *M*. Controls were included to rule out the possibility that RNA synthesis was inhibited because residual AAF carried along with the enzymes had inactivated the template used in the polymerase assays. First, additional untreated enzyme was added to aliquots of the

TABLE I. INHIBITION OF RNA SYNTHESIS IN ISOLATED RAT LIVER NUCLEI BY TREATMENT WITH AAF-DERIVATIVES *in vivo* OR *in vitro*.

Experiment	AAF derivative	Administration		Template	RNA polymerase activity[a] (pmoles)	(% of control)
1	N-OH AAF[b]	30 mg/kg,	0 hr[c]	Endogenous	72.0 ± 2.3	100
			1 hr	Endogenous	20.2 ± 8.2	28
			2 hr	Endogenous	21.2 ± 7.6	30
			12 hr	Endogenous	47.5 ± 3.6	66
			24 hr	Endogenous	54.0 ± 9.8	75
2	*N*-acetoxy AAF[d]		0	Endogenous	75.9 ± 1.0	100
			10^{-4} *M*	Endogenous	69.6 ± 5.2	92
			3×10^{-4} *M*	Endogenous	58.5 ± 8.5	77
			10^{-3} *M*	Endogenous	<1.0	2
			0	Poly d(AT)	17.8 ± 1.7	100
			10^{-3} *M*	Poly d(AT)	<1.0	6

[a] RNA polymerase assays were carried out in the presence of 1.6 m*M* $MnCl_2$ and 400 m*M* $(NH_4)_2SO_4$. Where poly d(AT) was used as template the mixture contained 40 μg/ml poly d(AT) and 10 μg/ml actinomycin D. Results represent means ± SD for liver nuclei from three animals.

[b] N-OH AAF was administered to rats *in vivo* as described in Methods.

[c] Time interval between N-OH AAF administration and sacrifice.

[d] Nuclei were treated *in vitro* with *N*-acetoxy AAF as described in Methods.

reaction mixtures after the first incubation and RNA synthesis again determined. In all cases the template showed little or no reduction in activity. As a second control, preincubation of enzyme was carried out with 9-[^{14}C]*N*-acetoxy AAF and after removing excess *N*-acetoxy AAF in the routine manner, DNA was incubated with the enzyme. Little or no binding of *N*-acetoxy AAF to the DNA was detected (data not shown). The results of Table II show that a reactive ester of AAF can directly inhibit transcription by inactivating either template or enzymes.

Binding of AAF derivatives to DNA and crude polymerases in vivo and in vitro. One way in which direct inactivation of template or enzyme almost certainly takes place is by the covalent binding of AAF residues to these macromolecules. Therefore, the amount of AAF bound when the enzyme or the template is inactivated *in vitro* with *N*-acetoxy AAF was compared with the AAF binding associated with inhibition of RNA synthesis *in vivo*. Treatment of rats *in vivo* with 30 mg/kg of N-OH AAF, a dose which drematically inhibits RNA synthesis (Table I), results in only about 1/100th as much AAF-binding to DNA as that required to inactivate the template *in vitro* (Table III). Thus, if direct template damage due to the binding of AAF is important in the inhibition of RNA synthesis *in vivo*, one must postulate preferential binding to particularly sensitive sites on the DNA. Binding of AAF to crude RNA polymerase (Table III) or highly purified RNA polymerases (Table IV) is also lower than the AAF binding required to produce substantial reduction in polymerase activity *in vitro* (Table III). As an illustration, incubation of crude RNA polymerase with 10^{-4} *M* *N*-acetoxy AAF causes only a slight reduction in RNA polymerase activity, but produces 9 times as much AAF binding to protein as that seen *in vivo* under conditions where RNA synthesis is inhibited to a greater extent.

Discussion. These results show that *N*-acetoxy AAF, a reactive ester of AAF is capable of inhibiting RNA synthesis *in vitro* by directly inactivating either the enzyme RNA polymerase or the template DNA. This work confirms the observation of Glazer *et al.* (17) that *N*-acetoxy AAF inactivates DNA as a template for rat liver RNA polymerases, and provides the first demonstration that an AAF derivative can directly inactivate RNA polymerases I and II. To our knowledge, this is

TABLE II. INHIBITION OF *in vitro* RNA SYNTHESIS BY PRETREATMENT OF DNA TEMPLATE OR ENZYME WITH *N*-ACETOXY AAF[a].

Experiment	Component Preincubated with *N*-acetoxy AAF	*N*-acetoxy AAF concentration (*M*)	Enzyme assayed	RNA polymerase activity (*p*moles)	RNA polymerase activity (% of control)
1	DNA	0	Crude	17.8 ± 1.4	100
		10^{-4}		16.2 ± 0.9	91
		10^{-3}		1.6 ± 1.1	9
	DNA	0	Polymerase I	19.1 ± 1.6	100
		10^{-4}		16.3 ± 1.3	86
		10^{-3}		4.1 ± 1.0	21
	DNA	0	Polymerase II	13.6 ± 0.8	100
		10^{-4}		9.9 ± 1.2	73
		10^{-3}		6.2 ± 0.1	46
2	Crude Polymerase	0	Crude	15.9 ± 1.7	100
		10^{-4}		14.5 ± 1.9	91
		10^{-3}		0.4 ± 0.3	3
	Polymerase I	0	Polymerase I	84.5 ± 5.1	100
		10^{-4}		81.9 ± 2.9	97
		10^{-3}		24.0 ± 2.3	29
	Polymerase II	0	Polymerase II	21.8 ± 1.6	100
		10^{-4}		15.9 ± 1.4	73
		10^{-3}		1.8 ± 0.8	8

[a] Preincubation of DNA or polymerases with *N*-acetoxy AAF and removal of unbound carcinogen were performed as described in Methods. Crude enzyme and Polymerase II were assayed in the presence of 100 m*M* $(NH_4)_2SO_4$ while Polymerase I was assayed in the presence of 40 m*M* $(NH_4)_2SO_4$. Results are expressed as means ± SD for three to five determinations.

TABLE III. BINDING OF AAF DERIVATIVES TO RAT LIVER DNA AND POLYMERASE PROTEIN *in vivo* AND *in vitro*.

A Binding to DNA

Labeled carcinogen	Method of administration	Amount or concentration of carcinogen	Carcinogen bound (nmoles/mg)
N-OH AAF	*In vivo*[a]	30 mg/kg	0.33 ± 0.06
N-acetoxy AAF	*In vitro*[b]	10^{-4} *M*	3.6 ± 0.4
N-acetoxy AAF	*In vitro*	10^{-3} *M*	55.8 ± 20.9

B Binding to protein

Labeled carcinogen	Method of administration	Amount or concentration of carcinogen	Protein fraction	Carcinogen bound (nmoles/mg)
N-OH AAF	*In vivo*[a]	30 mg/kg	Solubilized RNA Polymerase[c]	0.54 ± 0.13
N-acetoxy AAF	*In vitro*[b]	10^{-4} *M*	Solubilized RNA	4.8 ± 2.1
		10^{-3} *M*	Polymerase	29.0 ± 1.3
N-acetoxy AAF	*In vitro*	10^{-4} *M*	Polymerase I	1.8 ± 1.2
		10^{-3} *M*	Polymerase I	28.6 ± 7.1
N-acetoxy AAF	*In vitro*	10^{-4} *M*	Polymerase II	3.1 ± 0.6
		10^{-3} *M*	Polymerase II	35.1 ± 5.2

[a] 9[^{14}C]N-OH AAF (0.25 mC/mmole) was given to three rats by intraperitoneal injection. The animals were sacrificed 2 hr later (the time when AAF binding is maximal) and soluble enzyme and DNA were prepared. AAF-binding was determined as described in Methods. Results represent means plus or minus standard deviations from triplicate analyses.

[b] Purified DNA (300 μg/ml) or enzyme protein (100–200 μg/ml) was incubated for 1 hr at 25° in a medium containing 50 m*M* Tris–Cl (pH 7.7), 13% DMSO, and recently prepared *N*-acetoxy AAF. Processing was as described in Methods. Results represent means plus or minus standard deviations of three to five analyses.

[c] Crude nuclear enzyme solubilized as described by Roeder and Rutter (11) and centrifuged for 1 hr at 40,000 *g* to remove chromatin.

TABLE IV. BINDING OF AAF DERIVATIVES TO CRUDE AND PURIFIED RNA POLYMERASES *in vivo*[a]

Polymerase	Purification step	Enzyme specific activity (units/mg)	Carcinogen bound (nmoles/mg)
I & II	Crude nuclear extract	0.1	0.60
I	DEAE sephadex	1.1	0.60
I	Phosphocellulose	6.7	0.64
I	Sucrose gradient	N.T.[b]	0.50 ± 0.14[c]
II	DEAE sephadex	1.0	0.52
II	Phosphocellulose	5.9	0.71
II	Sucrose gradient	N.T.[b]	0.56 ± 0.10[c]

[a] 9[^{14}C]N-OH AAF (0.25 mCi/nmole) was given to each of 10 rats by intraperitoneal injection at dosage of 30 mg/kg. Two hr later the animals were sacrificed and N-OH AAF-inhibited RNA polymerases I and II were extensively purified from liver nuclei by methods described by Weaver *et al.* (18). After each purification step binding of AAF to polymerase protein was determined.

[b] Not tested because of insufficient material. Expected purification at this step is about fivefold.

[c] Mean ± SE for all gradient fractions containing protein.

the first time that direct inactivation of any enzyme by a derivative of this carcinogen has been shown. It seems probable that inactivation of a wide variety of enzymes may occur *in vivo* in an analogous manner and that such reactions may contribute significantly to the toxicity and perhaps the carcinogenicity of AAF.

The studies of carcinogen binding associated with inhibition of RNA synthesis by AAF derivatives *in vitro* and *in vivo* suggest, not surprisingly, that the situation *in vivo* is more complicated than that *in vitro*. The amount of AAF binding to DNA or protein seen when RNA synthesis is inhibited by N-OH AAF *in vivo* is considerably less than that required to inhibit enzyme or template activity by *N*-acetoxy AAF *in vitro*. This suggests that mechanisms may be operative *in vivo* which render selected portions of the template (for example, ribosomal genes) particularly sensitive to inactivation or which increase the sensitivity of polymerases to AAF. In particular, polymerases damaged by AAF may be subject to abnormally rapid turnover by cellular mechanisms. Whatever the processes responsible for this increased sensitivity, direct interaction of carcinogen with enzyme and template molecules is likely to be important in the inhibition of RNA synthesis by N-OH AAF *in vivo*.

Summary. The way in which the carcinogen *N*-hydroxy-*N*-2-acetylaminofluorene in-

hibits hepatic RNA synthesis was studied using the reactive ester *N*-acetoxy-*N*-2-acetylaminofluorene (*N*-acetoxy AAF). Preincubation of isolated nuclei with *N*-acetoxy AAF inhibited the ability of those nuclei to carry out RNA synthesis using endogeneous template or an exogenous template poly d(AT). Likewise, the template ability of DNA for RNA synthesis and the transcriptional capacity of RNA polymerases I and II were inhibited by preincubation of these macromolecules *in vitro* with *N*-acetoxy AAF. Inactivation of enzyme or template by *N*-acetoxy AAF was associated with and presumably caused by binding of AAF derivatives to these macromolecules. AAF binding required to inactivate polymerase or DNA *in vitro* was greater than that observed *in vivo* under conditions where RNA synthesis was inhibited to a comparable extent suggesting that additional mechanisms may operate *in vivo* to sensitize enzyme and template to inactivation by AAF binding.

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