EFFECTS OF ACROLEIN ON DNA SYNTHESIS *IN VITRO*

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1. Introduction

Acrolein or propenal (CH_2=CH-CHO), because of its biological effects and its presence in the gaseous phase of tobacco smoke, has been widely studied [1–9]. Injected into partially hepatectomized rats at concentrations higher than 1 mg/kg, acrolein was found to inhibit RNA and DNA synthesis in hepatic and pulmonary tissues [10].

However, the molecular mechanism of acrolein's effect on the nucleic acid synthesis in eukaryotic or prokaryotic cells has not yet been satisfactorily explained. It is known, that during *in vitro* RNA transcription [11], acrolein acts on RNA polymerase and not on DNA template, since the extent of transcription inhibition is unaffected by the amount of DNA added to the incubation medium. How acrolein interferes with the enzyme remains to be explained. Benedict and Stedman [12], from a study of the inhibitory action of cigarette smoke on three enzymes (lactic, alcohol and glucose 6-phosphate dehydrogenases), suggested that acrolein could deactivate the enzyme SH-groups by addition reactions. This assumption has also been put forward by Bilimoria and Nisbet [13] to account for the inhibition of *E. coli* L-asparaginase 2 by acrolein. To verify whether such a mechanism could apply to the enzyme responsible for nucleic acid synthesis and whether it could determine the variations of synthesis observed *in vivo*, we have studied the influence of acrolein on DNA synthesis *in vitro*.

To specify the possible role of the aldehyde on the enzymatic thiol groups, we used two different DNA polymerases: regenerating rat liver DNA polymerase and *Eschierichia coli* DNA polymerase I. The enzymatic activity of the first is associated with at least one functional thiol group [14], whereas the single cysteine residue of the second is not included in the active centre of the molecule [15]. From these studies we have found an inhibition of regenerating rat liver DNA polymerase by acrolein. Its site of action is located in the SH group(s) of the enzyme. On the other hand, *E. coli* enzyme was not inhibited by acrolein but activated.

2. Materials and methods

2.1. *Reagents*

Acrolein (Prolabo) was freshly distilled before use and diluted in distilled water to the desired concentration. 2-Mercaptoethanol was purchased from Eastman Kodak Co., Rochester, N.Y., USA. Iodoacetamide was supplied by Sigma Chemical Co.

2.2. *Substrates*

The following deoxyribonucleoside triphosphates used as substrates were: dATP, dCTP, dGTP, dTTP (Schwartz Bioresearch Inc., N.Y., USA), [^{3}H]dTTP (Amersham Radiochemical Centre, England, [α-^{32}P]dTTP (CEA, Saclay, France).

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Table 1

Acrolein action on the *in vitro* poly [d(A–T)·d(T–A)] duplication: effect on the regenerating rat liver DNA polymerase.

Preincubation (60 min at 38°)	Incubation (30 min at 38°)	
	Activity	Δ (%)
Enzyme + H_2O	5187	0
Enzyme + acrolein	2712	−48%
Template + H_2O	6447	0
Template + acrolein	6393	< 1%
Substrates + H_2O	4351	0
Substrates + acrolein	5551	27%

The polymerization reaction was started after preincubation (60 min at 38°) of acrolein (0.8 mM) with enzyme, template or substrates. The activity was expressed in pmoles of [^{3}H]dTMP incorporated by 1 ml of enzyme in 30 min.

2.3. *Templates*

We have used native or heat denatured calf-thymus DNA (Choay, France) and double stranded alternating copolymer poly [d(A–T)·d(T–A)] (Miles Inc. England).

2.4. *Enzymes*

DNA-dependent DNA polymerase of regenerating rat liver was prepared according to the method published elsewhere [16]. The purification procedure included the following steps: Fraction I, 105,000 *g* supernatant, Fraction II, ammonium sulfate precipitation (30 to 40% saturation), Fraction III, DEAE cellulose chromatography, Fraction IV, hydroxylapatite chromatography. Thus the DNA-dependent DNA polymerase has been purified 500 to 1,000-fold. In this paper, Fraction IV was used.

E. coli DNA polymerase I (Fraction VII) was purchased from Biopolymers Inc.

2.5. *Enzymatic assays*

The standard reaction mixture (0.25 ml) for the assay contained: 60 mM Tris-HCl (pH 7), 2.4 mM KCl, 3.4 mM $MgCl_2$, 200 μM deoxyribonucleoside triphosphates, 1 μCi [^{3}H]dTTP or 0.3 μCi [α-^{32}P]dTTP, 100 μM poly [d(A–T)·d(T–A)] or 200 μM DNA (native or denatured 5 min at 100°), 50 μl of enzyme (fraction IV of regenerating rat liver DNA polymerase or fraction VII of *E. coli* DNA polymerase I).

The assays for *E. coli* DNA polymerase I differed only with respect to the buffer in which the enzyme is stored (50 mM phosphate buffer, pH 7).

Table 2

Effects of acrolein concentration on the regenerating rat liver DNA polymerase activity.

Preincubation (60 min at 38°)	Incubation (30 min at 38°)							
	Acrolein (8×10^{-7} M)		Acrolein (8×10^{-6} M)		Acrolein (8×10^{-5} M)		Acrolein (8×10^{-4} M)	
	Activity	Δ (%)	Activity	Δ (%)	Activity	Δ (%)	Activity	Δ (%)
Enzyme + H_2O	4498	0	4498	0	4498	0	4498	0
Enzyme + acrolein	5999	+ 33	5838	+30	2729	−39	45	−99
Enzyme + substrates	481	0	481	0	481	0	481	0
Enzyme + substrates + acrolein	1343	+179	911	+89	418	−13	62	−87
Enzyme + 2-mercaptoethanol	3081	0	3081	0	3081	0	3081	0
Enzyme + 2-mercaptoethanol + acrolein	2826	−5	2669	−13	2729	−12	2767	−10

Enzyme: 50 μl of fraction IV; Substrates. dATP and [α-^{32}P]dTTP. Template: poly [d(A–T)·d(T–A)], 2-mercaptoethanol: 40 mM in buffer. The activity was expressed in pmoles of [α-^{32}P]dTMP incorporated by 1 ml of enzyme in 30 min.

Table 3
Opposite action of 2-mercaptoethanol to acrolein on the regenerating rat liver DNA polymerase.

1st Preincubation (30 min at 38°)	2nd Preincubation (30 min at 38°)	Incubation (30 min at 38°)	
		Activity	Δ (%)
Enzyme + H_2O	Buffer	2018	0
Enzyme + acrolein	Buffer	816	− 60
Enzyme + 2-mercaptoethanol	H_2O	5300	+160
Enzyme + acrolein	2-Mercaptoethanol	270	− 87
Enzyme + 2-mercaptoethanol	Acrolein	4144	+100

Template: poly [d(A−T)·d(T−A)]; acrolein: 0.8 mM in H_2O; 2-mercaptoethanol, 40 mM in buffer; enzyme: 50 μl of Fraction IV. The activity was expressed in pmoles of [α-^{32}P]dTMP incorporated by 1 ml of enzyme in 30 min.

Samples were incubated for 30 min at 38°. The acid insoluble fraction of an aliquot was precipitated on Whatman GF/C filter disc by 5% perchloric acid plus 2% sodium pyrophosphate, washed with 5% perchloric acid, ethanol and ether, dried, suspended in 5 ml scintillation fluid (4 g PPO, 0.1 g dimethyl-POPOP per ℓ toluene) and counted in a Packard scintillation spectrophotometer.

The enzymatic activity was expressed in pmoles of labelled deoxyribonucleoside monophosphate (dNMP) incorporated per ml of enzyme in 30 min at 38°.

3. Results

3.1. Action of acrolein on regenerating rat liver DNA polymerase

3.1.1. Effect on the enzyme

From a study of table 1, an acrolein–enzyme interaction seems to be fully responsible for the impaired replication *in vitro*, whereas, incubations of the substrates with acrolein slightly but reproducibly increase the enzyme activity and incubations of the template with acrolein do not affect the duplication.

3.1.2. Effects of acrolein concentration

When DNA polymerase was preincubated with increasing amounts of acrolein, the template duplication was either activated for low molarities or inhibited above $8 \cdot 10^{-5}$ M (table 2). A similar phenomenon was observed after a simultaneous preincubation of enzyme, substrates and acrolein. In the presence of 2-mercaptoethanol all acrolein effects were suppressed.

3.1.3 Antagonistic action of 2-mercaptoethanol and acrolein

Data in table 3 show that 2-mercaptoethanol and acrolein act oppositely on the enzyme, very likely on the cysteine group(s) essential for its catalytic activity.

Table 4
In vitro duplication of poly [d(A−T)·d(T−A)] by *E. coli* DNA polymerase I in the presence of acrolein.

Preincubation (60 min at 38°)	Incubation (30 min at 38°)					
	Acrolein (8×10^{-7} M)		Acrolein (8×10^{-6} M)		Acrolein (8×10^{-4} M)	
	Activity	Δ (%)	Activity	Δ (%)	Activity	Δ (%)
Enzyme + H_2O	18,200	0	18,200	0	18,200	0
Enzyme + acrolein	29,270	+60	27,800	+52	19,500	+ 7
Template + H_2O	29,900	0	29,900	0	29,900	0
Template + acrolein	38,300	+28	40,700	+36	41,900	+40
Substrates + H_2O	15,700	0	15,700	0	15,700	0
Substrates + acrolein	17,800	+13	18,300	+17	18,500	+18

Experimental conditions were as described in the text. Activity was expressed in pmoles of [^{3}H]dTMP incorporated by 1 ml of enzyme in 30 min.

Table 5

Lack of iodoacetamide effect on the *E. coli* DNA polymerase I activation produced by acrolein.

Preincubation	Incubation DNA polymerase activity	Δ (%)
Enzyme + H_2O	9,500	0
Enzyme + acrolein	12,900	+36
Enzyme + acrolein *	13,000	+37
Template + H_2O	12,200	0
Template + acrolein	16,750	+37
Template + acrolein *	18,600	+53
Substrates + H_2O	10,900	0
Substrates + acrolein	17,700	+63
Substrates + acrolein *	17,800	+64

Experimental conditions were as described in the text. Acrolein was 8×10^{-6} M. Activity was expressed in pmoles of [α-^{32}P]dTMP incorporated by 1 ml of enzyme in 30 min.

* Enzyme had been previously treated with iodoacetamide at a concentration of 10 mM for 4 min at 4°.

These data also demonstrate the irreversibility of the DNA polymerase inhibition induced by acrolein even in the presence of 2-mercaptoethanol.

3.2. *Action of acrolein on E. coli DNA polymerase I*

3.2.1. Effects of acrolein concentration

When *E. coli* DNA polymerase I is preincubated with increasing concentration of acrolein (8×10^{-7} M to 8×10^{-4} M), in contrast to the results obtained for regenerating rat liver DNA polymerase, the measured activities are always higher than those of the controls (table 4). In the presence of 2-mercaptoethanol, an *activation of E. coli enzyme by acrolein has not* been detected (unpublished results).

3.2.2. Acrolein effect on the enzyme, template and substrates

The results obtained after preincubation of acrolein with *E. coli* enzyme, template or substrates (table 4) indicate that the aldehyde can react with each of the present components of the medium and always favours the reaction of polymerization.

3.2.3. Iodoacetamide action

The action of acrolein on *E. coli* enzyme whose sulfhydryl group has been preliminarily blocked by iodoacetamide is in all cases similar to that observed for the enzyme whose sulfhydryl group has been left free (table 5). Thus the activation produced by acrolein does not result from the fixation of the aldehyde on the sulfhydryl group of *E. coli* DNA polymerase I.

4. Discussion

During the course of these experiments, it has been shown that acrolein has two effects on the *in vitro* DNA synthesis.

i) *The inhibitory action* of acrolein on partially purified DNA polymerase of regenerating rat liver is located in the sulfhydryl groups essential for the enzyme activity as recently pointed out by J.M. Rossignol et al. [14]. In fact, 2-mercaptoethanol and acrolein act oppositely on the enzyme. It has been reported [17] that 2-mercaptoethanol liberates the active sulfhydryl groups of the enzyme by reducing its disulfide bonds. Owing to the respective chemical properties of 2-mercaptoethanol and acrolein, it seems reasonable to conclude that acrolein, oxidizing the active thiol groups of the enzyme, inhibits the DNA polymerase, whereas 2-mercaptoethanol, reducing them, leads to an opposite effect:

$$E\text{–}SH + CH_2\text{=}CH\text{–}CHO \longrightarrow ES\text{–}CH_2\text{–}CH_2\text{–}CHO$$

This hypothesis is consistent with the fact that *E. coli* enzyme, devoid of SH groups in its active center, is not inhibited by acrolein but activated.

ii) *The activation* induced by acrolein, either at very low molarities as in experiments with regenerating rat liver DNA polymerase, or at higher molarities with *E. coli* enzyme, may be presumed to correspond to a different pattern either related to the oxidizing properties of acrolein double bond or to its aldehydic function.

For *E. coli* enzyme (table 4), it seems that acrolein likewise acts on the three components of the reaction. Since this enzyme lacks an SH group in its active center, only its positive action arises. On the other hand, for the mammalian enzyme the activation can only be detected as long as the active SH groups of the DNA

polymerase are not attacked by acrolein, i.e. at very low concentrations. Though it is still impossible to provide any definitive explanation of this phenomenon, it can be assumed that low concentrations of acrolein may form a complex with the substrates (H. Descroix et al. [18–19]). In this complex, both associated compounds could have lost their respective inhibitory properties with regard to the regenerating rat liver DNA polymerase. This hypothesis agrees with the activation observed after a simultaneous preincubation of enzyme, substrates and acrolein (table 2). However, in order to specify whether acrolein interacts with the templates or the substrates during the *in vitro* DNA synthesis, further experiments must be undertaken.

We have previously found that, *in vivo*, the inhibition of RNA and DNA synthesis occurred at acrolein concentrations higher than 1 mg/kg [10]. *In vitro* the RNA polymerase was also inhibited [11]. In this paper, we have shown that the regenerating rat liver DNA polymerase was inhibited *in vitro* at acrolein molarities between 2×10^{-5} M and 8×10^{-4} M.

It is well known that acrolein is the most abundant aldehydic compound in the vapor phase of cigarette smoke. If this constituent takes part in the carcinogenic process induced by tobacco, its action on the mammalian DNA and RNA polymerase may play a role in the carcinogenic process. Alacron has recently demonstrated that acrolein is produced during the oxidative degradation of antitumor agents (cyclophosphamide and isophosphamide). In view of the reactivity of this unsaturated aldehyde, it is suggested that it might be an effective cell-growth inhibitor [20]. This hypothesis agrees with the inhibition we observed both *in vivo* and *in vitro*.

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