The Effects of Nitrogen Mustard on DNA Template Activity in Purified DNA and RNA Polymerase Systems

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(Received November 27, 1967, and in revised form February 8, 1968)

SUMMARY

The effects of nitrogen mustard (HN2) on DNA and RNA synthesis in preparations utilizing purified DNA templates and polymerase enzymes have been investigated in order to more completely define the mechanism of action of antineoplastic alkylating agents at the molecular level. Pretreatment of native calf thymus DNA with 5×10^{-7} *M* HN2 produced a significant inhibition of DNA template activity in a purified RNA polymerase system from *Escherichia coli*. Concentrations of HN2 20–100 times higher were required to achieve an equivalent amount of inhibition of DNA template function utilizing a DNA polymerase system from *E. coli*. The template activities of both native and denatured DNA were inhibited by HN2. The quantity of alkylation of the DNA templates was determined by measuring the binding of ^{14}C-HN2, and the percent inhibition of DNA template activity was correlated with the number of alkylations per 10^4 nucleotide units. It was found that the template function of DNA as it exists in a nucleoprotein complex isolated from *E. coli* is relatively insensitive to treatment with HN2. The possible relationships of these findings to the cytotoxic effects of HN2 are discussed.

INTRODUCTION

Alkylating agents are known to produce alterations in the nuclei and nucleic acids of cells (1, 2). Evidence has accumulated which indicates that interaction with DNA may be causally related to the biological end effects of these drugs (3, 4). Several investigators have shown that the antimitotic and cytotoxic actions of the alkylating agent nitrogen mustard (HN2) are accompanied by a significant inhibition of DNA synthesis at concentrations which inhibit RNA and protein synthesis to a lesser degree (5–7). The cross-linking action of the difunctional alkylating agents has been suggested as the mechanism for the differential inhibition of DNA and RNA synthesis by HN2, since the degree of cross-linking between complementary DNA chains produced by HN2 may still allow partial unfolding, permitting RNA synthesis to occur whereas DNA synthesis could not (8). However, there is some evidence that the biological effectiveness of the mustards may not bear any quantitative relationship to the extent of alkylation of DNA and that attack on the DNA molecule per se may not be the crucial mechanism of action of the alkylating agents (9–12).

The present investigation was undertaken to determine what effect alkylation of purified DNA and deoxyribonucleoprotein has on their ability to act as templates for DNA and RNA synthesis in purified enzyme systems derived from *E. coli*.

METHODS

E. coli RNA polymerase was prepared from *E. coli* B cells (late log phase pur-

[1] Research supported by USPHS Grant CA-02992-11.

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chased from General Biochemicals) according to the method of Chamberlin and Berg (13). Fraction 4, obtained by chromatography on DEAE-cellulose, was used in the experiments. Enzyme dilutions were made with a solution containing 0.01 *M* Tris-HCl, pH 7.9, 0.01 *M* $MgCl_2$, 0.01 *M* 2-mercaptoethanol, 5×10^{-5} *M* EDTA, and 1 mg/ml of crystalline bovine serum albumin (BSA). The enzyme preparation was stored at 0–2° for several months. Under these conditions, the enzyme retained more than 80% of its activity for up to 2 weeks and 20–30% of the original activity after 2 months. The initial enzyme preparation had a specific activity of approximately 487 units per milligram; incorporation of 1.0 mμmole of ^{14}C-UMP into RNA per milligram of enzyme protein (under the conditions described below) was taken as one unit of enzyme activity. *E. coli* DNA polymerase was prepared from *E. coli* B (late log phase) according to the method of Richardson *et al.* (14). The enzyme preparation was taken through four purification steps, and fraction IV, obtained from ammonium sulfate fractionation, was utilized in these experiments. Fraction IV contained 4.5 mg/ml of protein. Enzyme dilutions were made with buffer containing 0.05 *M* Tris-HCl, pH 7.5, 0.1 *M* ammonium sulfate, 0.01 *M* 2-mercaptoethanol, and 1 mg/ml of BSA. A unit of enzyme activity was defined as that amount of enzyme which incorporated 10 mμmoles of total nucleotide into DNA in 30 min at 37° under the assay conditions described below. Fraction IV had a specific activity of 159 units per milligram of protein and was stored frozen for at least 6 months without loss of activity. Protein content was determined by the method of Lowry *et al.* (15).

DNA polymerase activity was routinely assayed in a 0.3-ml reaction mixture (unless otherwise indicated) containing: 30 μmoles Tris-HCl, pH 7.5; 2.3 μmoles $MgCl_2$; 0.8 μmoles 2-mercaptoethanol; 100 mμmoles each of dATP, dCTP, and dGTP; 20 mμmoles ^{14}C-TTP (0.025 μC); 11.2 μg enzyme protein (1.780 units); and 2.5–30 μg calf thymus DNA (Worthington). RNA polymerase activity was routinely determined in a 0.25-ml reaction mixture which contained the following; 10 μmoles Tris-HCl, pH 8.0; 2 μmoles $MgCl_2$; 3 μmoles 2-mercaptoethanol; 100 mμmoles each of ATP, CTP, and GTP; 50 mμmoles ^{14}C-UTP (0.025 μC); 10–50 μg enzyme protein (from 2–4 units); and 2.5–30 μg calf thymus DNA. In some experiments ^{14}C-ATP and ^{14}C-CTP were utilized as labeled precursors for the RNA polymerase assay. The reaction mixtures for both polymerase assays were incubated at 37° for 30 min, and the reactions were stopped with 3 ml of ice cold, 5% trichloroacetic acid. The precipitates were chilled for 10 min at 0° and then filtered on Millipore filters (0.45 μ pore size). The filters were washed with cold, 2.5% trichloroacetic acid three times and placed in scintillation counting vials. The filters were dried overnight in a desiccator. Toluene scintillator (10 ml) was then added, and the samples were counted on a Packard Tri-Carb liquid scintillation spectrometer. All assays of enzyme activity were determined in duplicate samples.

Both native and heat-denatured calf thymus DNA were assayed for template activity in the DNA and RNA polymerase reactions. DNA was denatured by heating the DNA solutions at 90–100° for 10 min and then rapidly cooling in ice water. In some experiments samples of native and denatured DNA templates (300 μg/ml) were preincubated with various concentrations of HN2 in 0.01 *M* Tris, pH 7.8, for 1–24 hr prior to assay of activity in the polymerase reactions. After 24 hr of incubation the pH of the reaction mixtures was between 7.1 and 7.8. The presence of unreacted HN2 in the preincubation mixtures was detected by the fluorimetric method of Mellett and Woods (16). It was found that incubation of the drug alone (10^{-3} *M*) in 0.05 Tris, pH 8.0, for 40 min resulted in at least 95% disappearance of the original compound.

The amount of binding of HN2 to the DNA preparations was determined by incubating DNA with various concentrations of ^{14}C-HN2 (sp. act. 0.064 mC/mmole) in 0.01 *M* Tris, pH 7.8, as described above.

The reactions were stopped with 3 ml of cold 5% trichloroacetic acid. The precipitates were chilled for 10 min at 0°, filtered on Millipore filters, washed, and counted as described above.

Ribosomes and the deoxyribonucleoprotein complex were prepared from extracts of *E. coli* B (grown in glucose-enriched Difco broth, harvested in the early log phase, and extracted by the method of Nirenberg and Matthaei) (17) by differential and gradient centrifugation as described by Shin and Moldave (18). The ribosomes were sedimented at 75,000 *g* for 3 hours, washed twice with standard buffer (0.01 *M* Tris-HCl, pH 7.8; 0.01 *M* magnesium acetate; 0.06 *M* KCl; and 0.006 *M* 2-mercaptoethanol) and then washed four times with a buffer containing 0.01 *M* Tris-HCl, pH 7.6; 0.5 *M* NH_4Cl; and 0.01 *M* magnesium acetate. The ribosomes were then suspended in standard buffer and frozen in small aliquots.

After removal of the ribosomes, the supernatant fraction was centrifuged at 75,000 *g* for 16 hours to obtain a nucleoprotein pellet containing DNA, RNA polymerase, and DNA polymerase activity. The pellets were resuspended in 0.01 *M* Tris-acetate buffer (pH 7.5) containing 0.01 *M* magnesium acetate and 0.007 *M* 2-mercaptoethanol. The suspension was centrifuged through a linear 8–30% sucrose gradient at 25,000 rpm (Spinco SW 25.1 rotor) for 24 hours. The gradient fractions containing RNA polymerase activity were frozen in small portions and thawed immediately before use.

RNA polymerase activity was assayed in the nucleoprotein preparations by determining the incorporation of ^{14}C-CMP or ^{14}C-UMP from the triphosphates into acid-insoluble product. Incubations were carried out for 30 min at 30° in a total volume of 0.5 ml and contained the following components: 15–20 mμmoles ^{14}C-CTP or ^{14}C-UTP; 100–150 mμmoles each of ATP, CTP, and GTP; 40 μmoles Tris-HCl, pH 7.5; 10 μmoles $MgCl_2$; 5 μmoles 2-mercaptoethanol; and 72 μg protein of the polymerase-DNA complex. In some experiments ribosomes (0.16 mg ribosomal protein) were added to the incubation mixtures prior to assay of RNA polymerase activity. The reactions were stopped with cold, 5% trichloroacetic acid. The precipitates were collected on Millipore filters, washed, and counted as above.

DNA polymerase activity in the nucleoprotein complex was assayed as above with the exception that 20 mμmoles of ^{14}C-dTTP and 100 mμmoles each of dATP, dGTP, and dCTP were utilized as precursors in a 0.45 ml reaction mixture. The effects of HN2 on the nucleoprotein complex and ribosomes were determined by preincubating the components with various concentrations of drug in 0.02 *M* Tris buffer, pH 7.8, for 60–120 min prior to assay of activity.

Nitrogen mustard and actinomycin D were donated by Merck and Company, Rahway, New Jersey. ^{14}C-Labeled nitrogen mustard (sp. act. 11.5 mC/mmole; 1,2-^{14}C) was obtained from Tracerlab and diluted with unlabeled HN2 prior to use. The radioactive precursors employed were purchased from New England Nuclear Corporation and had the following specific activities (mC/mmole): uridine-2-^{14}C 5′-triphosphate, 24.9; thymidine-2-^{14}C 5′-triphosphate, 30.3; cytidine-^{14}C (u.l.) 5′-triphosphate, 331; and adenosine-^{14}C (u.l.) 5′-triphosphate, 425.

Results

Characteristics of the DNA and RNA Polymerase Reactions

The data presented in Table 1 indicate that the DNA polymerase reaction requires template DNA and the presence of deoxyribonucleoside triphosphates. The amount of enzyme employed in these experiments appeared to be saturated by 15 μg of DNA template. The characteristics of the RNA polymerase reaction are presented in Table 2. This polymerase reaction required template DNA and ribonucleoside triphosphates, was inhibited by actinomycin D, and was essentially saturated above 15 μg of DNA.

TABLE 1

Characteristics of the DNA polymerase reaction

Reaction mixtures for experiment 1 contained 30 μg of native calf thymus DNA, 11.2 μg of DNA polymerase protein, 100 mμmoles each of dATP, dCTP, dGTP, and 5 mμmoles ^{14}C-TTP in addition to the other components specified in the Methods. Reaction mixtures for experiment 2 contained various amounts of DNA template and 20 mμmoles of ^{14}C-TTP; amounts of other constituents were the same as those described in the Methods. All incubations were for 30 min at 37°. In experiment 2, the control without DNA contained 81 cpm.[a]

Modifications	^{14}C-TMP (μμmoles) incorporated into DNA
Experiment 1	
Complete	3280
−DNA	92
−dATP	76
−dCTP	164
−dGTP	40
−dATP, dCTP, dGTP; + ATP, CTP, GTP	288
Experiment 2	
5 μg DNA	2758
10 μg DNA	4040
15 μg DNA	5179
30 μg DNA	4933

[a] In Tables 1–10, the activity (cpm) of samples without DNA template or zero time controls were subtracted before calculation of the data presented in each table.

Effect of HN2 on DNA Template Activity in the DNA and RNA Polymerase Reactions

Figure 1 depicts the effect of varying concentrations of HN2 on DNA synthesis using the purified DNA polymerase system. The template[3] activity of native calf thymus was inhibited by preincubation with 10^{-4} and 10^{-5} *M* HN2 for 4 hr at 37°. Inhibition occurred when the concentration of template was either subsaturating or saturating with respect to the polymerase enzyme (Table 1). Similar observations were made when the template activity of

[3] Since the products of the DNA polymerase reaction probably do not represent true replication of template DNA (see text), no attempt has been made to distinguish between the terms "template" and "primer" in this paper.

TABLE 2

Characteristics of the RNA polymerase reaction

Reaction mixtures contained various amounts of native calf thymus DNA, 100 mμmoles each of ATP, CTP, and GTP, 50 mμmoles of ^{14}C-UTP, and 10 μg of RNA polymerase protein. Amounts of other constituents were the same as those described in the Methods. All incubations were for 30 min at 37°. In experiment 2, the control without DNA contained 25 cpm.

Modifications	^{14}C-UMP (μμmoles) incorporated into RNA
Experiment 1	
Complete (+30 μg DNA)	4870
−DNA	50
−ATP	346
−CTP	578
−GTP	562
−ATP, CTP, GTP; +dATP, dCTP, dGTP	160
+ Actinomycin D (5 μg/ml)	300
Experiment 2	
2.5 μg DNA	989
5 μg DNA	1237
15 μg DNA	1800
30 μg DNA	2060

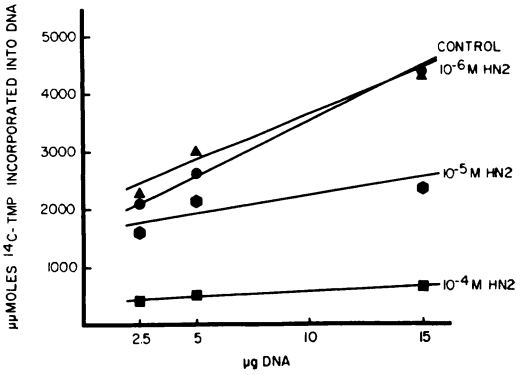

FIG. 1. *Effect of nitrogen mustard on the template activity of native DNA in the DNA polymerase reaction*

Calf thymus DNA (300 μg/ml) was preincubated with various concentrations of HN2 in 0.01 *M* Tris-HCl, pH 7.8, for 4 hr at 37°. Various amounts of alkylated DNA and nonalkylated DNA were assayed for template activity in the DNA polymerase system described in the Methods. A sample without DNA (190 cpm) was used as a control. This experiment was repeated 4 times with similar results.

native DNA was assayed in the RNA polymerase reaction (Fig. 2). However, the template activity of DNA for RNA synthesis was considerably more sensitive to the drug than template activity for DNA synthesis; inhibition was observed at 5×10^{-7} and 1×10^{-6} *M* HN2 in the RNA polymerase system.

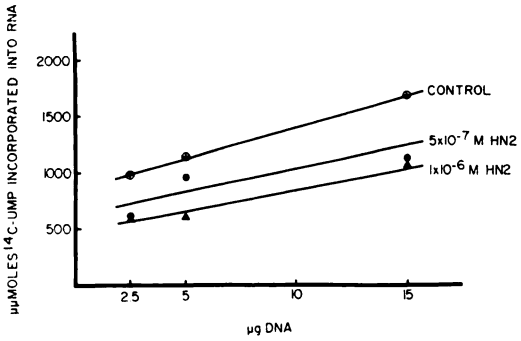

FIG. 2. *Effect of nitrogen mustard on the template activity of native DNA in the RNA polymerase reaction*

Calf thymus DNA (300 μg/ml) was preincubated with various concentrations of HN2 in 0.01 *M* Tris-HCl, pH 7.8, for 4 hr at 37°. Various amounts of alkylated and nonalkylated DNA were assayed for template activity in the RNA polymerase system described in the Methods. Each assay reaction mixture contained 10 μg of RNA polymerase. A sample without DNA (35 cpm) was used as a control. This experiment was repeated 3 times with similar results.

The effects of HN2 on the template function of native and heat-denatured calf thymus DNA are compared in Figs. 3 and 4. Both native and heat-denatured DNA were preincubated with alkylating agent for 4 hr prior to assay of activity. Template function of the treated samples was compared to control samples which had been preincubated under the same conditions. In all cases subsaturating amounts of template (2.5 μg) were employed in the polymerase reactions. In the DNA polymerase reaction denatured DNA was a more efficient primer than native DNA (2.97 mμmoles TMP incorporated by denatured *vs* 1.92 mμmoles by native DNA), and it appeared that the template function of the denatured primer was more sensitive to HN2. In the RNA polymerase

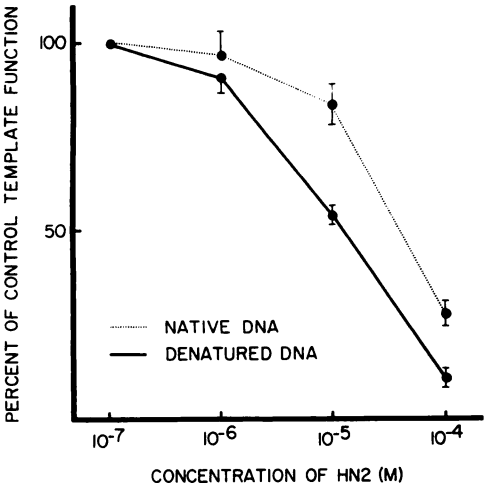

FIG. 3. *Effect of HN2 on the template activity of native and denatured DNA for DNA synthesis in a DNA polymerase system*

Both native and heat-denatured calf thymus DNA (300 μg/ml) were preincubated with HN2 in 0.01 *M* Tris-HCl, pH 7.8, for 4 hr at 37° prior to assay of template activity in the DNA polymerase system described in the Methods. Each assay reaction mixture contained 2.5 μg of either alkylated or nonalkylated DNA. The results are expressed as the mean percent change from control ±SE ($N = 3$–6).

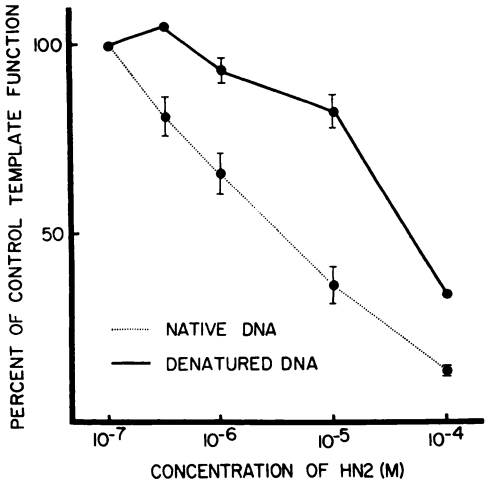

FIG. 4. *Effect of HN2 on the template activity of native and denatured DNA for RNA synthesis in a RNA polymerase system*

Preincubations and enzyme assays were carried out as described for Fig. 3.

reaction, however, native DNA was the preferred primer (2.88 mμmoles UMP incorporated by native *vs* 1.80 mμmoles by denatured DNA), and its template function for RNA synthesis was significantly more sensitive to the alkylating agent than that of denatured DNA. The data presented in Figs. 3 and 4 also indicate that the template function of native DNA for RNA synthesis is more sensitive to alkylation than the template function for DNA synthesis.

Kinetics of the DNA and RNA Polymerase Reactions Utilizing Alkylated and Nonalkylated DNA as Templates

The data presented in Fig. 5 show that incorporation of labeled triphosphate into DNA was linear over a 30-min period when native DNA was utilized as a template. Incorporation did not appear to be linear for longer than 10 min when denatured or alkylated templates were employed. The inhibitory effect of HN2 on template activity was constant when the duration of incubation exceeded 10 min.

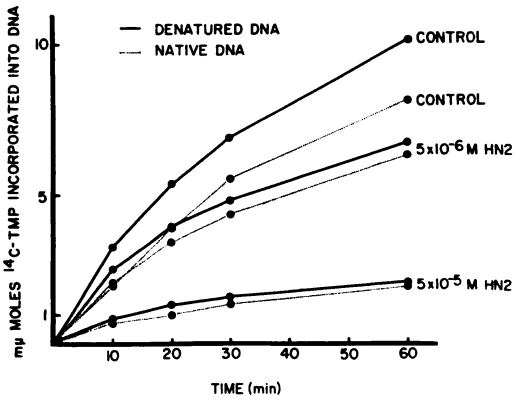

FIG. 5. *Kinetics of the DNA polymerase reaction using alkylated and nonalkylated DNA as templates*

Both native and denatured calf thymus DNA (300 μg/ml) were preincubated with various concentrations of HN2 in 0.01 *M* Tris-HCl, pH 7.8, for 4 hr. Ten micrograms of DNA was assayed for template activity in the DNA polymerase system described in the Methods. Zero time controls (80–200 cpm) were subtracted before calculation of millimicromoles incorporated. A repeat of this experiment gave similar results.

In the RNA polymerase reaction incorporation of labeled precursor was linear for 10 min (Fig. 6). There was not a substantial difference in the inhibitory effect of HN2 at any of incubation periods examined.

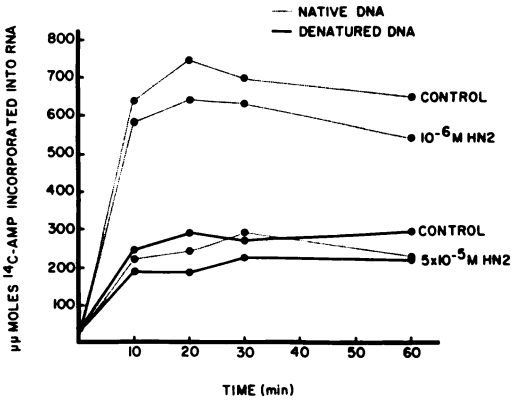

FIG. 6. *Kinetics of the RNA polymerase reaction using alkylated and nonalkylated DNA as templates*

Preincubations were carried out as described for Fig. 5. Ten micrograms of DNA was assayed for template activity in the RNA polymerase system described in the Methods. Zero time controls (50–200 cpm) were subtracted before calculation of micromicromoles incorporated. A repeat of this experiment gave similar results.

Effect of Treatment of Polymerases with HN2 on Their Enzymatic Activity

Neither of the polymerase enzymes were particularly sensitive to preincubation with HN2 (Tables 3 and 4). In both cases it took concentrations of 10^{-3} *M* HN2 to inhibit enzyme activity 30–35% below control levels. Thus the polymerase enzymes involved in nucleic acid synthesis do not appear to be nearly as sensitive to alkylation as the DNA template.

Relationship of the Amount of Alkylation of DNA to Its Template Activity

The amount of alkylation of DNA samples was determined by measuring the binding of ^{14}C-HN2 to DNA after preincubation with labeled alkylating agent for 1–24 hr (Table 5). The number of HN2 residues chemically bound per 10^4 nucleotide units was found to be concen-

TABLE 3
Effect of HN2 on DNA polymerase

DNA polymerase (120 μg/ml) was preincubated with HN2 in Tris buffer (0.01 *M*, pH 7.8) for 60 min at 37° prior to assay of enzyme activity. Enzyme assay mixtures (0.35 ml) contained 37.5 μg of native DNA, 11.2 μg of enzyme protein, 50 mμmoles each of dATP, dCTP, and dGTP, and 5 mμmoles of ^{14}C-TTP. Amounts of other constituents were the same as described in the Methods. Similar results were obtained in three additional experiments. All determinations were done in duplicate in experiment 1, and the data are expressed as mean ± range. A sample containing no DNA was used as a control in both experiments (10–30 cpm).

Modifications	^{14}C-TMP (μμmoles) incorporated
Experiment 1	
Enzyme preincubated without drug	1814 ± 87
Enzyme preincubated with HN2	
10^{-5} *M*	1863 ± 56
10^{-4} *M*	1607 ± 327
10^{-3} *M*	1176 ± 74
Experiment 2	
Enzyme not preincubated	1173
Enzyme preincubated with Tris buffer	936

tration dependent over the range of 10^{-7} to 10^{-4} *M* HN2, and maximal binding of HN2 occurred after 4 hr of reaction with DNA. However, after 24 hr of incubation, the amount of HN2 bound to native DNA was decreased below that observed after 4 hr. This suggests that alkylation products from the interaction of HN2 and DNA were hydrolyzed and released from the DNA molecule. Brookes and Lawley (1) have shown that a 35–45% loss of alkylated products, mostly in the form of 7-alkylguanines, occurs when mustard-treated DNA is incubated at pH 7.1 for 24 hr. This loss of alkylation products is similar in extent to the decrease in ^{14}C-HN2 bound to DNA observed after 24 hr of incubation (as compared to the maximal amount bound after 4 hr) in our experiments. Furthermore, there was a marked increase in the amount of OD_{260}-absorbable material passing through the Millipore filters after 24 hr of incubation of DNA with drug, suggesting a loss of alkylated bases from DNA rather than a simple hydrolysis of bound ^{14}C-HN2. It should be noted that the quantity of alkylation of DNA reported here is similar to, but somewhat greater than, that found by Kohn *et al.* (19), who incubated *Bacillus subtilis* DNA with ^{14}C-HN2 for 2 hr at 25° and pH 7.2. Our incubations were carried out at 37° and pH 7.5.

TABLE 4
Effect of HN2 on RNA polymerase

RNA polymerase (130 μg/ml) was preincubated with HN2 for 60 min at 37° in 0.01 *M* Tris, pH 7.8, prior to assay. Each assay reaction mixture (0.35 ml) contained 72.5 μg of native DNA, 13 μg of enzyme protein, 100 mμmoles each of ATP, CTP, and GTP, and 10 mμmoles of ^{14}C-UTP. Amounts of other constituents were the same as described in the Methods. The control without DNA contained 34 cpm.

Modifications	^{14}C-UMP (μμmoles) incorporated
Enzyme not preincubated	473
Enzyme preincubated without drug	403
Enzyme preincubated with HN2	
10^{-5} *M*	387
10^{-4} *M*	390
10^{-3} *M*	291

The data in Table 5 also indicate that denatured DNA bound less ^{14}C-HN2 than the native form and that no loss of bound ^{14}C-HN2 from denatured DNA occurred after 24 hr of incubation as compared to 4 hr. The fact that native DNA bound more ^{14}C-HN2 than denatured DNA suggests that the conformation of native DNA is such that it can more readily interact with HN2 than denatured DNA.[4]

The relationship of duration of preincubation with HN2 to the template activity of native DNA is demonstrated in Table 6. It was apparent that inhibition

[4] Samples of denatured and native DNA precipitated in trichloroacetic acid do not appear to pass through the Millipore filter. Thus the difference between the binding of HN2 to denatured DNA and to native DNA cannot be explained on this basis.

TABLE 5

Binding of ^{14}C-HN2 to calf thymus DNA

Each reaction mixture contained 300 μg/ml of calf thymus DNA in 0.01 *M* Tris, pH 7.8, and ^{14}C-HN2 (sp. act. 0.064 mC/mmole) in various concentrations. Incubations were carried out at 37°. At selected time intervals, aliquots containing 150 μg of DNA (0.5 ml) were withdrawn, acidified with cold 5% trichloroacetic acid, and filtered on Millipore filters as described in the Methods. The pH of the reaction mixtures after 24 hr of incubation was 7.1–7.2. The zero time controls, subtracted before data calculation, were 5, 11, 65, and 668 cpm for 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} *M* HN2, respectively, in experiment 1. The zero time controls in experiment 2 were 137 cpm with native and 35 cpm with denatured DNA. The greater amount of alkylation of native DNA compared to denatured DNA was observed in two other experiments in which different concentrations of HN2 were employed.

Expt. no.	Type of DNA	Incubation time (hr)	Number of ^{14}C-HN2 residues bound per 10^4 DNA nucleotide units. Concentration of ^{14}C-HN2 (*M*)			
			10^{-7}	10^{-6}	10^{-5}	10^{-4}
1	Native	1	0.5	2.9	28.0	248
		2	0.9	4.6	44.2	366
		4	1.0	5.9	56.4	457
		6	0.7	5.9	53.2	457
		24	0.3	3.2	31.5	193
2	Native	1	—	—	28.8	—
		4	—	—	55.3	—
		24	—	—	36.2	—
	Denatured	1	—	—	15.7	—
		4	—	—	33.0	—
		24	—	—	35.8	—

of DNA template function for DNA and RNA synthesis had achieved a near maximal level after only 1 hr of incubation of template with alkylating agent. It is somewhat surprising that there was only a 5–10% increase in inhibition between 1 and 4 hr of preincubation of template with drug since there was a 2-fold increase in the amount of ^{14}C-HN2 bound to native DNA over this time interval (Table 5). This can also be observed in Fig. 7, where the percent inhibition of DNA template activity has been plotted against the log of the number of alkylations per 10^4 nucleotides. This plot indicates that a 2-fold increase in the amount of alkylation of native DNA is necessary to achieve a 10% decrease in template function of native DNA. In addition this chart illustrates that it took six times more alkylations of native DNA to achieve a 50% inhibition of DNA synthesis than it did to produce the same inhibition of RNA synthesis. The data presented in Fig. 7 indicate that it would take approximately 25 alkylations per 10^4 nucleotides or 75 alkylations per molecule to produce a 50% inhibition of the template activity of one molecule of native calf thymus DNA (approximate mol. wt. 1×10^7 with average nucleotide mol. wt. 325) involved in RNA synthesis. If the plot in Fig. 7 is extrapolated to 100% inhibition of the template function of native DNA, it can be seen that it would take 500 alkylations per 10^4 nucleotide units to achieve complete inhibition. On this basis it would take 1500 alkylations with HN2 to completely inactivate a molecule of native calf thymus DNA.

Effect of Alkylation of DNA on the Base Content of RNA Product

The reactivity of the bases of the nucleic acids toward HN2 is not equal, but reaction with guanine bases seems to be

TABLE 6

Relationship of duration of incubation to the inhibition of DNA template function by HN2

DNA (300 μg/ml) was preincubated with HN2 in 0.01 *M* Tris, pH 7.8, at 37°. Control samples were preincubated under the same conditions but without HN2. Template activity of DNA (10 μg) was assayed in 0.3 ml reaction mixtures for DNA and RNA polymerase as described in the Methods. The millimicromoles of labeled precursor incorporated into nucleic acid for the controls were as follows: Experiment 1, 3.55 ^{14}C-TMP; experiment 2, 1.82 ^{14}C-TMP; experiment 3, 3.70 ^{14}C-CMP; experiment 4, 4.69 ^{14}C-UMP. All determinations were done in duplicate. Results are expressed as the percent decrease from controls ± range as compared to an averaged duplicate control. The controls varied ±4% in experiments 1–4. The controls without DNA contained 10–30 cpm.

Expt. no.	Polymerase	Conc. of HN2 (*M*)	Duration of preincubation (hr)	Percent decrease from control
1	DNA	5×10^{-5}	1	65 ± 10
			2	70 ± 2
			4	75 ± 3
			48	87 ± 1
2	DNA	5×10^{-5}	4	75 ± 3
			48	75 ± 2
			120	78 ± 1
3	RNA	1×10^{-6}	1	25 ± 4
			2	21 ± 1
			4	30 ± 1
4	RNA	5×10^{-6}	4	68 ± 2
			24	66 ± 1
			48	68 ± 1
			120	70 ± 3

favored (1). The reaction with guanine moieties may produce (a) steric hindrance of enzyme binding or base pairing due to insertion of bulky alkyl groups on the DNA chain, (b) interstrand or intrastrand cross-linking between guanine bases, (c) loss of alkylated guanine products from the DNA chain, and (d) chain scission (20). Thus the consequences of alkylation of DNA might produce an inhibition of the template function of DNA which would be more pronounced in guanine-rich areas of the DNA chain. Assuming that RNA polymerase may have multiple initiation sites along the DNA chain thereby allowing the enzyme to bypass alkylated sites (21), or that the enzyme can "slip" around alkylated portions of the DNA template, the inhibition of template function should be reflected by a greater decrease in the incorporation of CMP into RNA relative to other precursors whose incorporation is directed by pyrimidines. Furthermore, if alkylated guanine is misread as adenine as has been proposed (20), a relative increase in the incorporation of UMP might be expected.

To test this hypothesis, the template activity of subsaturating amounts of native calf thymus DNA was assayed in the RNA polymerase system after incubation of template with HN2 for 4 hr. The incorporation of ^{14}C-labeled ATP, CTP, and UTP into RNA was determined. The results expressed in Table 7 indicate that the incorporation of all three precursors was inhibited to a similar extent. These data suggest that RNA polymerase does not bypass alkylated guanine moieties to an extent sufficient to produce a differential inhibition of purine and pyrimidine precursors into RNA. It appears that the transcription of alkylated DNA stops when RNA polymerase reaches an alkylated site.

TABLE 7

Effect of alkylation of DNA by HN2 on the base content of RNA product

In each experiment native calf thymus DNA (300 μg/ml) was preincubated with various concentrations of HN2 for 4 hr at 37° in 0.01 *M* Tris, pH 7.8. Alkylated DNA (10 μg) was assayed for template activity in a 0.3 ml reaction mixture containing 20–50 μg RNA polymerase, 100 mμmoles of unlabeled nucleoside triphosphates, and 0.05 μC of labeled precursor as triphosphate. Other consitituents were as described in the Methods. All determinations were performed in duplicate, and the results are expressed as the percent decrease ± range as compared to an averaged duplicate control. The controls varied ± 3% in the experiments presented. The controls without DNA contained 10–30 cpm.

Expt. no.	Conc. of HN2 (*M*)	Percent inhibition of incorporation as compared to control. Labeled precursor		
		^{14}C-AMP	^{14}C-CMP	^{14}C-UMP
1	2.5×10^{-7}	7 ± 3	8 ± 2	—[a]
	1×10^{-6}	30 ± 3	30 ± 1	—
2	5×10^{-7}	18 ± 2	26 ± 3	—
3	5×10^{-7}	13 ± 1	24 ± 0	20 ± 2
	5×10^{-6}	59 ± 0	64 ± 0	51 ± 10
4	2.5×10^{-6}	38 ± 1	38 ± 2	39 ± 1

[a] Not examined.

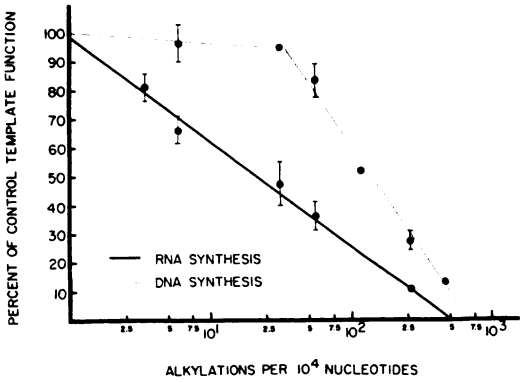

FIG. 7. *Relationship of the extent of alkylation of native DNA to the inhibition of DNA template function for nucleic acid synthesis in the DNA and RNA polymerase systems*

The data presented in this figure are a combination of the results presented in Figs. 3 and 4, Table 5, and additional experimental results obtained as described in the Methods.

Effect of HN2 on the Template Function of a Deoxyribonucleoprotein Complex Isolated from E. Coli

In order to examine more closely the effects that HN2 might have on DNA template activity when DNA is present in its more natural form as a nucleoprotein complex, the deoxyribonucleoprotein preparation from *E. coli* described by Shin and Moldave (18) was utilized. This preparation contains endogenous DNA template and RNA polymerase activity. We have determined that the nucleoprotein pellet also contains DNA polymerase activity (Table 8). In addition, an augmentation of RNA synthesis by ribosomes, suggesting a function of ribosomes in the control of polyribonucleotide synthesis (22), can be demonstrated in this preparation (Table 9).

The template activity of the nucleoprotein complex for DNA synthesis was relatively insensitive to pretreatment with the alkylating agent (Table 8); a concentration of 10^{-3} *M* HN2 produced only a slight inhibition (15%) of DNA synthesis. Similarly, RNA synthesis supported by the nucleoprotein preparation was not inhibited until a concentration of 10^{-3} *M* HN2 was attained; however, at this concentration RNA synthesis was inhibited 85% (Table 9). In addition, the stimulation of RNA synthesis observed after the addition of highly washed ribosomes (no DNA present) was not significantly affected by preincubating either the nucleoprotein or the ribosomes with HN2 prior to assay of

TABLE 8
Effect of HN2 on DNA synthesis directed by a deoxyribonucleoprotein complex from Escherichia coli

The nucleoprotein complex (NP) (32 μg DNA and 72 μg protein) was isolated from *E. coli* as described in the Methods and preincubated with HN2 in 0.02 *M* Tris (0.3 ml), pH 7.2 to 7.8 at 37° prior to assay of template activity. Assay mixtures were as described in the Methods. Data are expressed as mean ± range for duplicate samples. Zero time controls contained 25–50 cpm.

Modifications	^{14}C-TMP (μμmoles) incorporated
Experiment 1	
NP not preincubated	1230
NP preincubated without drug for 120 min	400 ± 6
NP preincubated with HN2 for 120 min	
10^{-5} *M*	470 ± 2
10^{-4} *M*	560 ± 25
10^{-3} *M*	342 ± 15
Experiment 2	
NP not preincubated	972 ± 31
NP preincubated for 60 min without drug	859 ± 28
NP preincubated for 60 min with 5×10^{-4} *M* HN2	737 ± 3

RNA polymerase activity. Actinomycin D markedly inhibited the synthesis of RNA in the presence of ribosomes.

We had noted earlier (23) that preincubation of *E. coli* ribosomes with 5×10^{-3} *M* HN2 produced a 66% inhibition of their ability to participate in poly U-directed protein synthesis and a 50% decrease in their capacity to bind ^{3}H-poly U. Apparently the functional sites of the ribosome involved in the direction of protein synthesis and binding of mRNA are distinct from those involved in the enhancement of RNA polymerization.

Table 10 illustrates the amount of alkylation of nucleoprotein that occurs after treatment with ^{14}C-HN2. From these data a comparison can be made between the quantity of alkylation of the DNA present in the nucleoprotein complex and the quantity of alkylation of an equivalent

TABLE 9
Effect of HN2 on RNA synthesis directed by a deoxyribonucleoprotein complex from Escherichia coli

The nucleoprotein complex (32 μg DNA and 72 μg protein) was preincubated with HN2 for 120 min (experiment 1), or 60 min (experiment 2). ^{14}C-UTP was utilized as precursor in experiment 1, and ^{14}C-CTP was used in experiments 2, 3, and 4. Reaction mixtures were as described in the Methods. In some experiments 0.16 mg ribosomal protein was added (with or without preincubation with HN2 for 60 min) prior to assay of polymerase activity. Determinations were done in duplicate where indicated. A repetition of experiments 2 and 3 produced similar results. Zero time controls contained 50–60 cpm.

Modifications	Labeled (μμmoles) precursor incorporated
Experiment 1	
NP not preincubated	370
NP preincubated for 120 min without drug	116 ± 15
NP preincubated with HN2	
1×10^{-5} *M*	104 ± 4
1×10^{-4} *M*	122 ± 11
1×10^{-3} *M*	18 ± 3
Experiment 2	
NP not preincubated	468
NP preincubated for 60 min without drug	264
NP preincubated + ribosomes	628 ± 24
NP preincubated with HN2 + ribosomes	
5×10^{-6} *M*	572 ± 8
5×10^{-5} *M*	570 ± 19
5×10^{-4} *M*	549 ± 20
Experiment 3	
NP	468
NP + ribosomes preincubated without drug	765 ± 48
NP + ribosomes preincubated with HN2	
5×10^{-5} *M*	763 ± 80
5×10^{-4} *M*	727 ± 11
5×10^{-3} *M*	708 ± 16
Experiment 4	
NP + ribosomes	420
NP + ribosomes + actinomycin D (20 μg/ml)	49

TABLE 10

Binding of ^{14}C-HN2 to deoxyribonucleoprotein, calf thymus DNA, and bovine serum albumin

Each reaction mixture (0.3 ml) contained either *Escherichia coli* deoxyribonucleoprotein complex (32 μg DNA, 72 μg protein), calf thymus DNA (32 μg), bovine serum albumin (72 μg), or both calf thymus DNA (32 μg), and BSA (72 μg) together with various concentrations of ^{14}C-HN2 (623 cpm/mμmole) in 0.02 *M* Tris, pH 7.8. Incubations were carried out at 37°. The procedure for the determination of amount of bound ^{14}C-HN2 is described in the Methods. After incubation with ^{14}C-HN2, some of the nucleoprotein samples were heated to 80–90° for 15 min to remove the DNA prior to filtration on Millipore filters. The amount of ^{14}C-HN2 bound per 10^4 nucleotides was determined from the differences in the quantity of ^{14}C-HN2 bound before and after heating.

Preparation incubated	Conc. of HN2 (*M*)	Duration of incubation (hr)	Cpm ^{14}C-HN2 bound	Cpm ^{14}C-HN2 bound after heating	^{14}C-HN2 residues bound per 10^4 nucleotide units
Deoxyribonucleoprotein	10^{-4}	1	323	255	11
	10^{-4}	2	425	298	22
	10^{-4}	4	420	—[a]	—
	10^{-3}	1	2194	1375	132
	10^{-3}	2	3314	2452	139
	10^{-3}	4	2978	—	—
Calf thymus DNA	10^{-4}	2	1332	87	200
	10^{-3}	2	7251	616	1070
Bovine serum albumin	10^{-4}	2	540	567	—
	10^{-3}	2	3396	3416	—
Calf thymus DNA + bovine serum albumin	10^{-4}	2	1409	544	139
	10^{-3}	2	9908	4351	893

[a] Not determined.

amount of purified DNA in the presence of the same amount of protein as found in the nucleoprotein complex. It can be seen that the conformation of the nucleoprotein complex must provide for the protection of DNA from attack by HN2, since addition of an equivalent amount of protein (bovine serum albumin) to a solution containing purified calf thymus DNA did not protect the DNA from alkylation to nearly the same extent. In fact, purified DNA seemed to compete for binding of HN2 more effectively than protein, an observation which most likely reflects the higher density of sterically available nucleophilic moieties in DNA.

From observing the amount of alkylation of DNA in the nucleoprotein and determining the percent change from control template function for purified DNA which had been alkylated with the same amount of HN2 per 10^4 nucleotides (Fig. 7), it was expected that pretreatment with 10^{-3} *M* HN2 would produce a 45% inhibition of DNA synthesis (observed = 15% inhibition) and a 75% decrease in RNA synthesis (observed = 85%). Likewise, preincubation with 10^{-4} *M* HN2 should have produced no effect on DNA synthesis (observed = 0%) and a 45% decrease in RNA synthesis (observed = 0%). Thus it appears that equivalent amounts of alkylation do not produce the same decrease in template function of *E. coli* DNA, as it exists in a nucleoprotein complex, and purified calf thymus DNA.

DISCUSSION

In an attempt to define more completely the mechanism of action of the antineoplastic alkylating agent at the molecular level, the effects of HN2 on DNA and RNA synthesis in preparations utilizing purified DNA templates and polymerase enzymes have been investigated.

Our data indicate that pretreatment of

calf thymus DNA with concentrations of HN2 as low as $5 \times 10^{7} M$ (3.4 alkylations per 10^{4} nucleotides) will produce a significant inhibition of DNA template activity in a purified RNA polymerase system. It took concentrations of HN2 20–100 times higher to achieve an equivalent amount of inhibition of DNA synthesis utilizing the DNA polymerase system. Chmielewicz *et al.* (24) have also reported that the template function of calf thymus DNA for RNA synthesis is more sensitive to alkylation with HN2 than its template function in DNA synthesis. However, these investigators found no significant inhibition of RNA synthesis until DNA was preincubated with $4 \times 10^{-4} M$ HN2, and the priming activity of DNA, measured in a regenerating rat liver DNA polymerase system, did not show any decrease upon treatment with HN2 (25). It should be pointed out that the RNA polymerase system used by these investigators was from a different source (*Micrococcus lysodeikticus*) and apparently had a somewhat different specific activity than the *E. coli* RNA polymerase employed in the experiments reported here. Furthermore, the DNA polymerase utilized by these authors was taken from the 106,000 *g* supernatant without further purification before use (25). This may explain the greater sensitivity of DNA template function to alkylation observed in our experiments.

Our observations conflict with the data obtained in intact cells since it has been demonstrated that DNA synthesis is more sensitive to HN2 treatment than RNA synthesis (5–7). It may be that there is a more specific inhibition of synthesis of mRNA's involved in the synthesis of DNA replicating enzymes as suggested by Chmielewicz *et al.* (24) or that the DNA polymerase enzyme preparation employed is not the actual DNA replicating enzyme. There has been recent evidence to suggest that, in fact, the DNA polymerase preparation utilized by us and by other workers is not the true DNA replicase but a repair enzyme (26, 27). Thus it may well be that the studies done to date, utilizing cell-free systems or purified enzymes, do not reflect the actual sensitivity of DNA synthesis to HN2 and other agents.

The template activities of both native and denatured DNA were inhibited by HN2. Denatured DNA was more sensitive to HN2 than native DNA in the DNA polymerase reaction, in which denatured DNA is the preferred template, and native DNA was more sensitive in the RNA polymerase reaction where the native form is the more effective primer. In both cases, then, the more active form of DNA is the one whose activity is more susceptible to an inhibitory action of the drug. It should be noted that one possible explanation for the lower sensitivity of denatured DNA as a template in the RNA polymerase reaction is that denatured DNA binds 25 times more RNA polymerase than does native, helical DNA (21). Conceivably, then, it would take many more alkylations to inhibit the function of denatured DNA as a template for RNA synthesis.

The mechanism for the inhibition of DNA template function by HN2 may involve: (a) interstrand or intrastrand cross-linking by the drug, (b) steric hindrance of enzyme binding or base pairing at alkylated sites, (c) deletion of alkylated bases, (d) chain breakage, (e) denaturation or changes in the secondary structure of DNA. Brookes and Lawley (1, 28) have reported that bifunctional alkylating agents inactivate genetic material primarily by interstrand cross-linking of double-stranded DNA and that intrastrand cross-linking does not occur to a significant extent. However, Yamamoto *et al.* (29) have suggested that alkylation with HN2 inactivates single-stranded phage DNA by forming intrastrand cross-links between neighboring coils on the same, single helix. They also observed that double-stranded DNA phages were inactivated somewhat more rapidly than single-stranded phages. Our data indicate that the template capacity of denatured DNA is sensitive to alkylation, and in the case of the DNA polymerase reaction it is even more sensitive than native DNA. This evidence suggests that interstrand cross-linking between the strands of the double

helix in DNA is not the only and perhaps not the most important mechanism of inhibition of DNA template activity. Kohn and Green (30) reported that nitrogen mustard-cross-linked *Bacillus subtilis* DNA retains a high degree of activity for genetic transformation; transforming activity remains even in molecules with at least two cross-links. The latter data also suggest that interstrand cross-linking per se does not abolish the biological activity of DNA. It was estimated (Fig. 7) that it would take approximately 75 alkylations per molecule of calf thymus DNA to produce a 50% inhibition of RNA synthesis and 1500 alkylations per molecule to produce a 100% decrease in RNA synthesis. Kohn *et al.* (19) have determined that the binding of approximately 25 molecules of HN2 to DNA is required to produce 1 effective cross-link. On this basis, it would take 3 cross-links per molecule to produce a 50% inhibition of DNA template function and 60 cross-links to produce a complete inactivation.

The second possible mechanism for the decreased template ability of alkylated DNA is that a steric effect might be produced by the insertion of bulky alkyl groups on the chain. Our evidence indicating that there is no apparent difference in the relative incorporations of AMP, CMP, and UMP into RNA in the presence of alkylated template suggests that this factor may be important. From this observation it seems that the RNA polymerase can move along the template until it reaches an alkylated site, and then transcription stops. In addition, we have found (Table 6) that the deletion of 35–45% of the alkylated bases from DNA, which was evident after 24 hours of incubation with HN2, does not produce a significant change in the inhibition of DNA template activity. In this regard, Weiss and Wheeler (31) have concluded, on the basis of their evidence and that of others (32–34) for decreased template activity of irradiated DNA as a primer for RNA synthesis, that base destruction or deletion is not a major factor in the loss of priming activity, since it is probable that at a site of base damage an available complementary or noncomplementary base could be inserted.

In regard to the other potential mechanisms for inhibition of template function, it is not likely that either chain scission or other measurable changes in the physicochemical properties of the DNA occur at concentrations at which a significant inhibition of template function is obtained (24). Therefore, of the potential mechanisms listed above it seems that (a) and (b) are the most likely to be involved in the impairment of template function of alkylated DNA.

The activity of the polymerase enzymes themselves appears to be quite resistant to alkylation. Smellie *et al.* (35) have shown that Lettré-Ehrlich DNA polymerase is resistant to 10^{-4} *M* HN2 *in vitro*. Papirmeister (36) reported calf thymus DNA polymerase to be sensitive to 10^{-3} *M* sulfur mustard, but that *E. coli* DNA polymerase was resistant to this concentration. Our data indicate that *E. coli* DNA and RNA polymerase are resistant up to a concentration of 10^{-3} *M* HN2.

In intact cells it has been demonstrated that DNA synthesis is more sensitive than RNA synthesis, but the question whether the effects on nucleic acid synthesis are due primarily to an action on the template, on enzyme activity, or possibly on an "initiator" of DNA synthesis (37) is not settled. Goldstein and Rutman (38) have shown that treatment of mice bearing the Lettré-Ehrlich ascites tumor with doses of HN2 sufficient to effect a significant extension of host survival time produced a marked inhibition of *in vitro* thymidine-^{3}H incorporating capacity by the ascites cells. This inhibition resulted from a reduction in both the enzymatic and primer activities associated with DNA synthesis, but the effect on the enzyme system was quantitatively the most important. The data of Wheeler and Alexander (39) indicate that alkylating agents interfere with the *de novo* synthesis of ribonucleotides and the conversion of ribonucleotides to components of DNA in drug-sensitive hamster plasmacytomas. Tomisek *et al.* (40) have reported a decrease in DNA polymerase and thymi-

dylate kinase in plasmacytomas treated with the alkylating agent cyclophosphamide. All the effects on enzymes, however, could conceivably reflect an inhibition of DNA-directed nucleic acid synthesis. Our data indicate that the template activity of purified DNA is remarkably sensitive to alkylation with concnetrations of HN2 which can be achieved *in vivo* with tumor-inhibitory dose levels.

On the other hand, the experiments of Ord and Danielli (41), using amoebae, indicate that the components of the cytoplasm involved in DNA synthesis and mitosis are more sensitive to alkylation than the nucleus of the cell. The significance of this observation is heightened by the fact that Prescott and Goldstein (42) have recently demonstrated in amoebae that the cytoplasm has a significant degree of control over nuclear DNA synthesis. Our experimental evidence indicates that the template function of DNA as it exists in a nucleoprotein complex is relatively insensitive to treatment with HN2. Although it is not surprising that the presence of protein in nucleoprotein protects the DNA from alkylation (Table 10), these data suggest that DNA, as it exists as nucleoprotein in the cell, may not be the most sensitive site to alkylation and that other sites of action may also be important for the cytotoxic effect of HN2.

To summarize the effects of HN2 on the biosynthetic mechanisms in the *E. coli* cell-free system, we have compared the present data with data reported earlier on protein synthetic mechanisms (23). The various components of the biosynthetic machinery are listed (Table 11) in order of decreasing sensitivity, and the "threshold" dose of HN2, i.e., the lowest concentration which will produce a significant inhibition as compared to controls, is also given. It can be seen that the template activity of native calf thymus DNA for RNA synthesis is the most sensitive. The "amino acid-polymerizing" enzymes present in the S-100 (100,000 *g*) fraction are next in sensitivity, followed by the template activity of DNA in DNA synthesis. The ability of synthetic messenger RNA's to act as templates for protein synthesis is inhibited at 10^{-5} *M*. The template activity of the nucleoprotein complex, the ability of the ribosomes to support protein synthesis, and the activities of DNA and RNA polymerase are inhibited at 10^{-3} *M*. Aminoacyl-tRNA synthetases are the least sensitive of the components examined, not being significantly affected until a concentration of 5×10^{-3} *M* is employed.

TABLE 11
Sensitivity of the components of the Escherichia coli B cell-free system to nitrogen mustard

Component	"Threshold" concentration (*M*)
Native DNA in RNA synthesis	5×10^{-7}
S-100 enzymes for amino acid polymerization	1×10^{-6}
Native DNA in DNA synthesis	5×10^{-6}
Messenger RNA (synthetic)	1×10^{-5}
Nucleoprotein complex Ribosomes DNA polymerase RNA polymerase	1×10^{-3}
S-100 enzymes for aminoacyl-tRNA synthesis	5×10^{-3}

ACKNOWLEDGMENT

The authors wish to thank Linda Galligher for her excellent technical assistance.

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