Interaction of Nitrogen Mustard with Polyribonucleotides, Ribosomes, and Enzymes Involved in Protein Synthesis in a Cell-Free System

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SUMMARY

The effects of nitrogen mustard (HN2) on synthetic polyribonucleotides, ribosomes, and enzymes involved in protein synthesis in an *Escherichia coli* cell-free system were investigated. It was found that HN2 decreased the coding capacity of three synthetic polymers: poly A, poly U, and poly C. Poly C and poly U were more sensitive to HN2 than poly A. Ribosomes and protein synthesizing enzymes (100,000 *g* supernatant) obtained from *E. coli* cells were preincubated with drug and assayed for activity in the cell-free polypeptide synthesizing system. The effect of nitrogen mustard on the ribosomes was concentration dependent; lower concentrations (10^{-6} to 10^{-4} M) stimulated polypeptide synthesis while higher concentrations (10^{-3} M) inhibited synthesis. Protein-synthesizing enzymes were inhibited by nitrogen mustard at both high and low concentrations. These results suggest possible sites of action of the biological alkylating agent HN2 at the level of genetic code translation of messenger RNA into protein.

INTRODUCTION

Nitrogen mustard, *N*-methyl-bis(β-chloroethyl)amine, produces a variety of effects on biological systems (1). Among the actions of this agent is the ability to depress protein synthesis both *in vivo* and *in vitro* (1). *In vitro* studies (2) also have shown that nitrogen mustard, at a concentration below that which inhibits protein synthesis, can stimulate amino acid incorporation into protein in an *E. coli* cell-free system.

The discovery of a cell-free protein synthesizing system (3) which can utilize a synthetic polyribonucleotide (i.e., poly U) as a messenger RNA (mRNA) provides an excellent system for the study of the interactions of nitrogen mustard (HN2) with the components (i.e., mRNA, ribosomes, etc.) involved in the mechanism of protein synthesis. This type of study presents an opportunity to gain much additional information about the mechanism of action of alkylating drugs and the biological effects of alkylation. Using this experimental approach, Ludlum *et al.* (4) have shown that methylation of polyadenylic acid (poly A) by methyl methanesulfonate markedly inhibits the ability of the polymer to code for polylysine synthesis in an *E. coli* cell-free system. Methylation occurred at the N-1 position on the adenine ring and reduced the ability of adenine to base-pair with uracil. This suggests a possible explanation for the loss of coding ability of the methylated polymers. Similarly, Wahba *et al.* (5) have shown that replacement of 10% of the bases in polyuridylic acid (poly U) with methyluracil inhibits the incorporation of phenylalanine into polypeptide. Recently it has been demonstrated by Abell *et al.* (6) that sulfur mustard reacts

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at the 5′-phosphate of the chain-terminal nucleotide in poly U, resulting in decreased activity of the polynucleotide to direct phenylalanine incorporation. These workers suggested that esterification of the terminal 5′-phosphate of poly U may prevent the binding of aminoacyl-tRNA to the poly U-ribosome complex and result in loss of template activity.

In the present investigation the effects of nitrogen mustard on synthetic polyribonucleotides, ribosomes, and enzymes involved in protein synthesis in an *E. coli* cell-free system were studied.

METHODS

Escherichia coli (strain B) were grown at 37° in a glucose enriched Difco broth (7) and harvested in the early log phase of growth. The cells were disrupted, extracted, and S-30, ribosomal, and S-100 fractions prepared by the method of Nirenberg and Matthaei (3). The extraction was done with standard buffer (5× weight of washed cells) containing 0.01 M Tris-HCl, pH 7.8, 0.01 M magnesium acetate, 0.06 M KCl, and 0.006 M β-mercaptoethanol.

For experiments utilizing synthetic messenger RNA's, fresh S-30 was incubated for 40 min at 30° to reduce the amount of endogenous *E. coli* messenger RNA. The reaction mixture contained the following in micromoles per milliliter unless otherwise specified: 50 Tris-HCl, pH 7.9; 15 magnesium acetate; 60 KCl; 9 β-mercaptoethanol; 2 ATP; 3 phosphoenolpyruvate, sodium salt (PEP); 0.3 guanosine 5′-triphosphate (GTP); 0.1 each of 20 amino acids; and 0.02 mg pyruvate kinase (Calbiochem). After incubation, the reaction mixture was dialyzed against 60 volumes of standard buffer at 5° overnight. The dialyzing buffer was changed once during the course of dialysis. The incubated S-30 (Incub S-30), washed ribosomes (W-Rib), and supernatant at 100,000 *g* (S-100) fractions were stored in small aliquots at −15°. Protein content of Incub S-30, S-100, and W-Rib fractions was determined by a modification of the method of Lowry (8). Optical density at 260 mμ of the W-Rib fraction was determined in a Beckman DU spectrophotometer.

Synthetic polyribonucleotides (poly U, poly A, and poly C) were obtained from Miles Chemical Co. and used without further purification. Alkylation of the synthetic polymers was carried out by incubation at 37° for 60 min with various concentrations of nitrogen mustard (Merck and Co.) in 0.05 M Tris-HCl, pH 7.5–8.0. The presence of unreacted HN2 in the preincubation mixture was detected by the fluorimetric method of Mellett and Woods (9). This method measures the amount of active alkylating groups in nitrogen mustard. It was found that incubation of the drug alone (10^{-3} M) in 0.05 M Tris, pH 8.0 for 40 min resulted in at least 95% degradation of the active compound. This solution (0.1 ml) had no observable effect on protein synthesis when added to a poly A-directed polylysine synthesizing system (2.1 ml). It was, therefore, concluded that the amount of biologically active alkylating agent which was remaining after preincubation and which could be transferred to the reaction mixtures in the aliquots employed (0.1 ml) was insignificant. In the drug-polymer reaction mixtures (0.3 ml), the concentrations of poly U, poly A, and poly C were 1, 2, and 6.8 mg/ml, respectively. Using these concentrations, 0.1-ml aliquots of the solutions containing HN2-treated polymers were conveniently assayed for coding capacity in the cell-free polypeptide synthesizing system described below.

The coding capacity of synthetic polyribonucleotides (both alkylated and nonalkylated) was determined by measuring the incorporation of ^{14}C-amino acids into acid-insoluble protein using the Incub S-30 fraction (poly U, poly C, and poly A directed the incorporation of ^{14}C-labeled phenylalanine, proline, and lysine, respectively). The reaction mixture contained the following in micromoles per milliliter unless otherwise noted: 50 Tris-HCl, pH 7.8; 15 magnesium acetate; 70 KCl; 4.2 β-mercaptoethanol; 2.5 PEP; 1.0 ATP; 0.3 GTP; 0.1 μC of ^{14}C-amino acid; 20 μg pyruvate

kinase; 2.5–3.0 mg Incub S-30 protein; and either 50 μg poly U, 100 μg poly A, or 340 μg poly C. Total volume of the reaction mixtures was 2.1 ml. The mixtures were incubated at 37° for 40 min. In experiments measuring phenylalanine-^{14}C incorporation as directed by poly U, the reactions were stopped with 3 ml of 10% trichloroacetic acid (TCA), and the precipitates washed with 5% TCA. For poly A-directed lysine-^{14}C incorporation, 0.5 mg of carrier polylysine (General Biochemicals) was added to the reaction mixture, and the reactions immediately stopped with 2.5 ml 10% TCA and 4 ml of 0.5% Na_2WO_4–5% TCA, pH 2. Washings were done with 0.25% Na_2WO_4–5% TCA, pH 2. For poly C directed proline-^{14}C incorporation, 0.5 mg of carrier polyproline (General Biochemicals) was added, the reactions were stopped with 5 ml 20% TCA, and the protein samples were washed with 20% TCA. The washing procedure and the removal of nucleic acids from the precipitates were performed by a modification of the method of Siekevitz (10). The purified protein samples were filtered on Whatman 50 filter paper disks (16 mm in diameter), removed from the paper, dried, weighed, and placed into 20-ml screw-cap counting vials. The protein (4–5 mg) was dissolved in 1 ml of 1 M Hyamine hydroxide (Packard Instruments Co.) by heating at 75° for 30–60 min. Toluene phosphor scintillator (10 ml) was added, and the samples were counted in a Packard Tri-Carb scintillation spectrometer. Results were calculated as counts per minute (cpm) per milligram of protein, and all determinations were done in duplicate. The radioactive amino acids (Nuclear Chicago Corporation) used had the following specific activities (mC/mmole): L-phenylalanine-U-^{14}C 7.0; L-proline-U-^{14}C 120; and L-lysine-U-^{14}C 150.

Ribosomes (W-Rib) and enzymes (S-100) were incubated at 37° for 40 min with various concentrations of HN2 in standard buffer, pH 7.0–7.8. Each reaction mixture (0.6 ml) contained 1.5–2.0 mg W-Rib protein or 2–3 mg of S-100 protein. Control ribosomes and enzymes were preincubated in standard buffer without drug. After incubation, the fractions were placed in crushed ice until they were assayed for activity in the cell-free system. The activity of HN2-treated ribosomes and enzymes was determined by measuring the poly U-directed incorporation of phenylalanine-^{14}C into protein. The reaction mixture for protein synthesis was identical to the one described above with the exception that 1.5–2.0 mg of W-Rib protein and 2.0–3.0 mg of S-100 protein were employed in place of Incub S-30 protein in the reaction mixtures, which had a final volume of 2.3 ml.

The binding of ^{3}H-poly U (Miles Chemical Company) (specific activity 27.3 mC/mmole) to *E. coli* ribosomes was assayed according to the method of Barondes and Nirenberg (11). In some cases, ^{3}H-poly U and ribosomes were pretreated with nitrogen mustard as described above. The reaction mixture contained 0.5–1.0 mg W-Rib protein and 0.2 μC of ^{3}H-poly U in 0.45 ml of standard buffer, pH 7.8. The mixtures were made at 0–2° and 0.2 ml was immediately layered on top of a 5-ml linear sucrose gradient (5–20% in standard buffer). The samples were centrifuged at 27,500 rpm in either a SW39 or SW50 rotor (Spinco) for 3 hr, and 3-drop samples were collected after piercing the bottoms of the tubes. The 3-drop samples were diluted with 1 ml H_2O and assayed for radioactivity and optical density at 260 mμ.

The binding of aminoacyl-^{14}C-tRNA to HN2-treated messenger-ribosome complexes was studied. Phenylalanyl-^{14}C-tRNA (specific activity 30,850 cpm/mg tRNA) was prepared from *E. coli* "stripped" tRNA (General Biochemicals) and phenylalanine-^{14}C (specific activity 222 mC/mmole) by the method of von Ehrenstein and Lipmann (12). The incubation mixture (0.5 ml) contained the following, in micromoles unless otherwise specified: 25 Tris-HCl, pH 7.6; 10 magnesium acetate; 50 KCl; 40 μg poly U; 8 OD_{260} units of *E. coli* ribosomes; 100 μg of phenylalanyl-^{14}C-tRNA containing 3085 cpm. After incubation at 25° for 30 min, the ribosomes were col-

lected, washed, and assayed for radioactivity as described by Nirenberg and Leder (13).

RESULTS

Effect of HN2 on the Coding Capacity of Synthetic Messenger RNA

Preincubation of the synthetic polyribonucleotides poly U, poly C, and poly A with HN2 prior to their use in the cell-free system derived from *E. coli* cells resulted in a dose-dependent inhibition of their ability to direct the incorporation of phenylalanine, proline, and lysine, respectively, into protein. Table 1 shows the effect of preincubation with various concentrations of HN2 on the coding capacity of poly U. Nitrogen mustard similarly produced an inhibition of the ability of poly C and poly A to direct protein synthesis, although the coding activity of poly A was remarkably less sensitive to the alkylating agent than the coding ability of poly U and poly C. Figure 1 summarizes the effects of HN2 on the coding capacity of the three synthetic polynucleotides. Poly U and poly C were similar in their response to nitrogen mustard in that the first significant inhibition of coding capacity was observed at 1×10^{-5} M. On the other hand, the coding ability of poly A was relatively resistant to the action of the drug up to a concentration of 1×10^{-3} M. Therefore, at the lower concentrations of HN2 the pyrimidine-containing polymers appeared to be more sensitive than the purine-containing polynucleotide.

TABLE 1

Effect of preincubation with HN2 on the ability of polyuridylic acid to direct phenylalanine-^{14}C incorporation

Alkylation of poly U with nitrogen mustard and subsequent assay of template activity are described in the Methods.

Modifications	Activity (cpm/mg protein)
Poly U (50 μg/ml) preincubated with:	
No drug	8,489
Nitrogen mustard	
5×10^{-6} M	8,457
1×10^{-5} M	7,387
1×10^{-4} M	6,420
1×10^{-3} M	4,370
5×10^{-3} M	2,296
Deproteinized at zero time	30

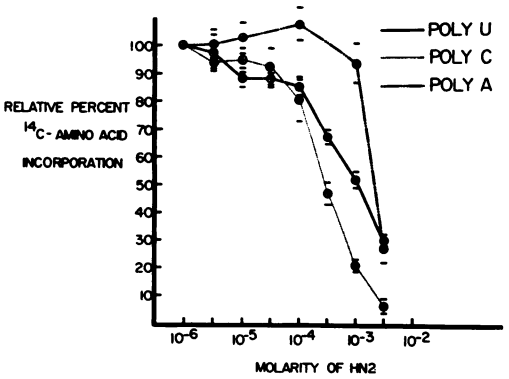

FIG. 1. *Effect of HN2 on the template activity of synthetic polyribonucleotides*

All polymers were preincubated with nitrogen mustard for 60 min at 37° and assayed for template activity as described in the Methods. Nonalkylated poly A and poly C consistently incorporated 1500–2000 cpm/mg protein of ^{14}C-labeled lysine and proline, respectively. Residual endogenous *E. coli* messenger RNA activity (100–300 cpm/mg) present in the Incub S-30 fraction was determined in each experiment and subtracted from the total radioactivity to give an accurate measurement of the coding capacity of the synthetic polymer. The results are expressed as the mean percent change from control ± SE.

Effect of Preincubation of Ribosomes and S-100 Enzymes with HN2 on Their Ability to Participate in Protein Synthesis

Washed ribosomes from *E. coli* were pretreated with various concentrations of nitrogen mustard prior to use in a system utilizing poly U-directed phenylalanine-^{14}C incorporation into protein as an index of ribosomal function. The effect of HN2 on ribosomal activity is shown in Table 2. Concentrations of HN2 from 10^{-6} to 10^{-4} M produced a 17–22% stimulation of phenylalanine-^{14}C incorporation compared to control. However, elevation of the concentration of HN2 in the preincubation medium

TABLE 2

Effect of HN2 on the activity of E. coli ribosomes in poly U-directed polyphenylalanine-^{14}C synthesis

Alkylation of *E. coli* ribosomes with nitrogen mustard was carried out as described in the Methods. Repetition of this experiment ($n = 5$) indicated the drug-induced stimulation of amino acid incorporation to be significant ($p < 0.05$).

Modifications	Activity (cpm/mg protein)
Complete	25,989
− Ribosomes	1,998
− S-100 enzymes	746
− ATP, GTP, PEP, and pyruvate kinase	600
Ribosomes preincubated with HN2:	
1×10^{-6} M	30,438
1×10^{-4} M	31,670
1×10^{-3} M	15,995
5×10^{-3} M	8,754
Deproteinized at zero time	609

to 1×10^{-3} M or greater produced inhibition of cell-free protein synthesis. Thus, it appears that the stimulation of protein synthesis noted previously in the S-30 cell-free extracts treated with HN2 (2) is a property of the ribosomes. Furthermore, concentrations of drug which depressed protein synthesis in the S-30 extracts also depressed the ability of the ribosomes to function in polypeptide synthesis.

TABLE 3

Effect of HN2 on the activity of E. coli protein-synthesizing enzymes (S-100 fraction) in poly U-directed polyphenylalanine-^{14}C synthesis

Alkylation of the enzymes with nitrogen mustard was carried out as described in the Methods. Additional experiments ($n = 4$) gave similar results.

Modifications	Activity (cpm/mg protein)
Complete	11,173
− S-100 enzymes	554
S-100 enzymes preincubated with HN2:	
10^{-6} M	9,754
10^{-5} M	9,389
10^{-4} M	9,096
10^{-3} M	6,689
Deproteinized at zero time	88

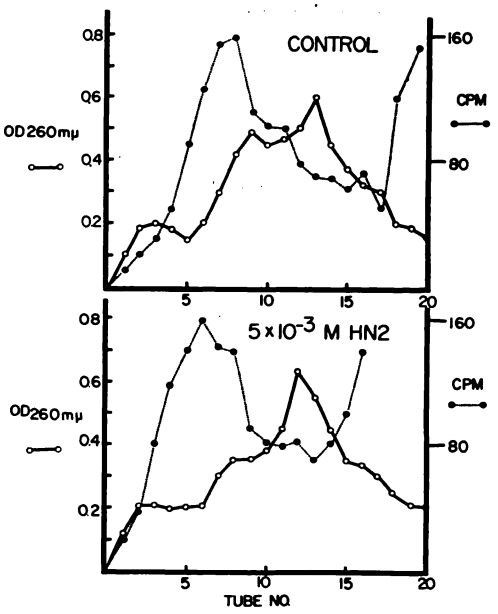

FIG. 2. *Binding of alkylated ^{3}H-poly U to E. coli ribosomes*

^{3}H-poly U (0.2 μC) was preincubated with 5×10^{-3} M HN2 for 60 min at 37° in a 0.2 ml reaction mixture containing 0.05 M Tris, pH 7.5. The binding capacity of alkylated ^{3}H-poly U for ribosomes was determined as described in the Methods.

Preincubation of the S-100 supernatant fraction containing the protein-synthesizing enzymes resulted in depression of polyphenylalanine synthesis at all concentrations of drug tested (10^{-6} to 10^{-3} M) as shown in Table 3.

The Effects of HN2 on the Binding of ^{3}H-Poly U to Ribosomes

The binding of ^{3}H-poly U to ribosomes was examined using both alkylated[2] ^{3}H-poly U and alkylated[2] ribosomes. Upon mixing ^{3}H-poly U with ribosomes, binding

[2] It has been asssumed for purposes of discussion in this paper that HN2 interacts with the components of the protein-synthesizing scheme by the chemical mechanism of alkylation, although we have not isolated alkylated derivatives of each of these components. Therefore, we use the term "alkylation" to refer to an interaction of HN2 with a given component when such an interaction may produce an observed biochemical alteration of function of that component.

occurred immediately at 0–2°. Unbound ^{3}H-poly U remained at the top of the gradient. Any period of incubation at higher temperatures produced less binding than was observed with the initial mixing at 0–2°. After incubation at 37° for 10 min there was no ^{3}H-poly U bound to the ribosomes, presumably because of rapid destruction of the messenger by ribosomal nucleases (11). Figure 2 shows the effect of alkylation of ^{3}H-poly U upon its binding to ribosomes. Alkylation of ^{3}H-poly U with a concentration of HN2 (5×10^{-3} M) which inhibited coding capacity 70% produced no observable decrease in its binding capacity for ribosomes as compared with control.

Figure 3 shows the ability of HN2-treated ribosomes to bind ^{3}H-poly U. Ribosomes preincubated with a concentration of HN2 which inhibited polypeptide synthesis 66% (see Table 2) were less able to bind ^{3}H-poly U than control. Thus, the inhibition of polypeptide synthesis noted when ribosomes were pretreated with HN2 appears to be due to inability of the ribosomes to bind the synthetic messenger RNA. In addition, the effects of preincubation with 10^{-6} M HN2, a concentration which stimulated polypeptide synthesis, on the ability of isolated ribosomes to bind ^{3}H-poly U was investigated. Figure 4 shows that there was no significant change from control at this concentration. Thus, it seems that, although the mechanism of stimulation of protein synthesis by HN2 involves the ribosomal fraction, it is not at the level of the binding of messenger RNA to ribosomes.

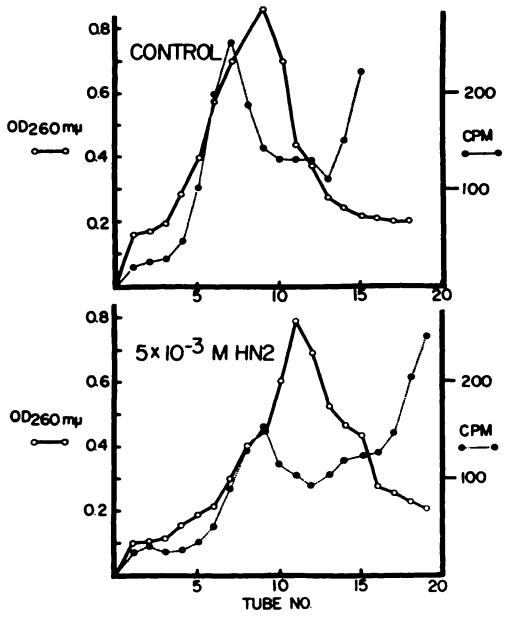

FIG. 3. *Binding of ^{3}H-poly U to ribosomes pretreated with 5×10^{-3} M HN2*

Ribosomes were preincubated with 5×10^{-3} M HN2 and subsequently assayed for ability to bind ^{3}H-poly U as described in the Methods.

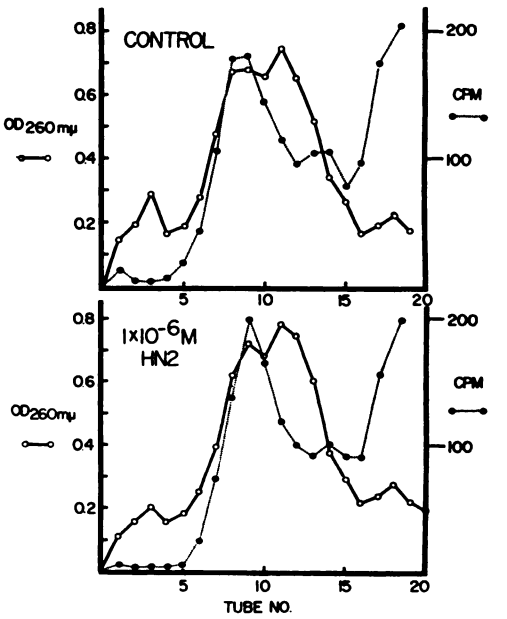

FIG. 4. *Binding of ^{3}H-poly U to ribosomes pretreated with 1×10^{-6} M HN2*

Ribosomes were preincubated with 1×10^{-6} M HN2 and subsequently assayed for ability to bind ^{3}H-poly U as described in the Methods.

Effect of HN2 on the Binding of Phenylalanyl-^{14}C-tRNA to a Poly U-Ribosome Complex

Table 4 shows the effect of nitrogen mustard on the binding of phenylalanyl-^{14}C-tRNA to a poly U-ribosome complex. Alkylation of the poly U template with 5×10^{-3} M HN2 produced no significant

decrease in binding of phenylalanyl-^{14}C-tRNA. On the other hand, alkylation of ribosomes with 5×10^{-3} M nitrogen mustard produced a substantial decrease (approximately 45%) in binding of phenylalanyl-^{14}C-tRNA. Reaction of ribosomes with concentrations of HN2 (10^{-6} and 10^{-4} M) which stimulated polypeptide synthesis did not produce an increase in binding of phenylalanyl-^{14}C-tRNA to the ribosomes.

TABLE 4

Effect of nitrogen mustard on the binding of phenylalanyl-^{14}C-tRNA to a messenger-ribosome complex

Reaction mixtures (0.5 ml) were prepared as described in the Methods and incubated for 30 min at 25°. Alkylation of poly U and *E. coli* ribosomes with nitrogen mustard was carried out as described in the Methods.

Modifications	Cpm ^{14}C-Phe-tRNA bound
Experiment 1	
No template	82
Poly A	84
Poly U	1,359
Poly U alkylated (5×10^{-3} M HN2)	1,302
Poly U + ribosomes alkylated (5×10^{-3} M HN2)	726
Experiment 2	
Poly U	1,623
Poly U + ribosomes alkylated with HN2:	
1×10^{-6} M	1,585
1×10^{-4} M	1,520
1×10^{-3} M	1,408
5×10^{-3} M	941

DISCUSSION

In order to explain the *in vivo* effects of nitrogen mustard on protein synthesis at the molecular level, the effects of the drug upon the components involved in protein synthesis, namely messenger RNA, ribosomes, and enzymes have been systematically examined.

It has been reported (14, 15) that alkylating agents interact with the bases in DNA and RNA and that these sites of reaction are critical in producing the biological effects of these agents. Brookes and Lawley (14) have shown that the prime target for alkylation in nucleic acids is the N-7 position of guanine. The reactivity of bases in nucleic acids to alkylation appears to occur in the following order: guanine > adenine > cytosine ≫ thymine or uracil (16). With the polymers utilized in this study a theoretical prediction of the sensitivity of the polymers to alkylation, as evidenced by biological template activity and based on purine or pyrimidine base reactivity, should be: poly A > poly C ≫ poly U. However, our data show the following order of sensitivity to HN2: poly C ⩾ poly U > poly A. Therefore, it appears that there must be some other site of alkylation in these polymers in addition to the bases in order to explain the observed data.

Alternative sites for alkylation in RNA are the diesterified and terminal phosphate groups. It has been shown that methylation of RNA esterifies the internal phosphates to yield phosphate triesters which hydrolyze spontaneously, breaking the polymer into fragments by chain fission (17). Jones *et al.* (18) have found that short fragments ($n < 50$) of poly U and poly C have little capacity to direct the synthesis of acid-insoluble polypeptides. On the other hand, short chains of poly A *were* capable of producing acid-insoluble polylysine. Thus, it is conceivable that cleavage of poly U and C into ineffective template fragments by phosphate alkylation could be the mechanism responsible for the loss of template activity due to treatment with nitrogen mustard. In addition, Abell *et al.* (6) have reported that sulfur mustard alkylates the 5′-terminal phosphate of poly U and that this is responsible for the observed loss of template activity of alkylated poly U. However, using poly U as a model messenger RNA, we have shown that alkylation of the polymer with a concentration of HN2 which inhibits the coding capacity 70% does not interfere with the binding of the polymer to ribosomes, nor does it interfere with the binding of phenylalanyl-^{14}C-tRNA to a ribosome-alkylated poly U complex. Thus, if HN2

alkylates poly U on the 5′-terminal phosphate as shown by Abell *et al.* for sulfur mustard, this interaction appears to have no inhibitory effect on the ability of the polymer to initiate protein synthesis.

On the other hand, there is evidence that base alkylation may also play a role in template inactivation. Ludlum *et al.* (4) have shown that the methylation of poly A with high concentrations of methyl methanesulfonate (0.1–0.2 M) inhibits the coding capacity of the polymer and also its ability to complex with poly U, i.e., base-pair. This same type of mechanism could be involved in the HN2-inactivation of poly A, poly C, and poly U at high concentrations of the drug.

There is a possibility that cross-linking of the polyribonucleotides by HN2 may contribute to the loss of template activity produced by the alkylating agent. The "degree of helicity" for polyribonucleotides in buffered solutions at 37° appears to follow the order poly C > poly A > poly U, with poly U existing in the form of randomly coiled chains (19). It would be expected that the possibility of cross-linking of the polymers during alkylation would increase with increasing helicity. The fact that poly U is more sensitive to alkylation than poly A, which may attain a high degree of secondary structure, suggests that cross-linking of these polymers is not the critical mechanism of template inactivation. However, cross-linking may be involved in the inactivation of poly C, which may attain a high degree of helicity and which is apparently the polymer most sensitive to the action of HN2.

Nitrogen mustard has been shown to be carcinogenic in experimental animals (20). The stimulation of amino acid incorporation into protein produced by ribosomes which had been pretreated with 10^{-6} to 10^{-4} M HN2 and also seen in crude S-30 extracts (2) suggests that this agent has a tendency to produce effects which disrupt the regulation of protein synthesis, providing a possible starting point for carcinogenic changes. Although ribosomes which have been treated with 10^{-6} M nitrogen mustard can stimulate polypeptide synthesis, these ribosomes were not able to bind ^{3}H-poly U or phenylalanyl-^{14}C-tRNA to any greater extent than nonalkylated ribosomes. A possible explanation of the enhanced activity of the ribosomes may be the inhibition of ribosomal nucleases by HN2, giving rise to a longer lifetime of messenger RNA and thus more polypeptide synthesis. We have observed (unpublished observations) that nitrogen mustard can increase the lifetime of ^{14}C-labeled RNA in an S-30 extract at 37°.

Although the concentrations of HN2 employed in some of the experiments are somewhat higher than could be achieved *in vivo* at therapeutic dose levels, the data presented here indicate the level of sensitivity of an *in vitro* cell-free system to the "acute" effects of an agent whose actions are known to be more evident in a population of growing, dividing cells over a longer period of time. Indeed, the antimitotic effect of HN2 is perhaps the most sensitive action of the drug (21). However, that nitrogen mustard can produce effects on the protein-synthesizing machinery in an artificial "cell sap" in which the factors of absorption, distribution, and cellular transport are eliminated indicates that these drug effects are at least potential mechanisms of action *in vivo*. Furthermore, assuming equal distribution of a therapeutic, tumor-inhibitory dose of HN2 (2 mg/kg, i.e., one-half of the LD_{50} dose by intraperitoneal injection) (22) in a mouse bearing the Ehrlich ascites tumor, a concentration of 1×10^{-5} M could be achieved. Allowing for partial concentration of the drug in certain tissues, a concentration somewhat larger than this could be achieved locally *in vivo*.

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