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Comparative Effects of Methyl- and Ethylnitrosourea on DNA Directing Cell-Free DNA-Dependent Synthesis of β-Galactosidase

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SUMMARY

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An *in vitro* DNA-directed system for synthesis of β-galactosidase was used to study the effects of methylnitrosourea (MNU) and ethylnitrosourea (ENU) on function of the DNA template. Both MNU and ENU inhibited formation of β-galactosidase activity when added to the complete system at the beginning of incubation; this inhibition increased with increasing concentrations of either MNU or ENU. When template DNA was exposed to MNU or ENU before use in the protein synthesis system, β-galactosidase synthesis was greatly reduced. Under comparable conditions, MNU was more inhibitory than ENU. Incubation of DNA with MNU or ENU resulted in DNA alkylation, which increased linearly with the concentration of nitrosourea over the range 0–1% alkylated nucleotides. MNU was shown to act both as an alkylating agent and, presumably via its degradation product NCO^-, as a carbamoylating agent, but alkylation was far more extensive than carbamoylation after 10 min at 37°C and pH 8.0. The degree of inhibition of β-galactosidase synthesis was directly related to the extent of total alkylation, irrespective of whether MNU or ENU was allowed to react with template DNA. No difference in genotoxicity between these two agents was observed which could be ascribed to differences in sites of alkylation. Carbamoylation of DNA by exposure to KNCO did not inhibit synthesis of β-galactosidase, although KNCO was highly inhibitory when added to the complete system.

INTRODUCTION

Both MNU[1] and ENU are strong mutagens and carcinogens (1). They react with DNA, chiefly by alkylation but also to a limited extent by carbamoylation (2–8). These reactions result in altered secondary structure and perturbation of base-pairing interactions that may affect various aspects of DNA function, including replication and transcription (5, 8–11). ENU is a weaker electrophile than MNU and tends to yield proportionately fewer ring *N*-alkylated bases and more *O*-alkylated products such as O^6-ethylguanine and phosphotriesters (8, 12). Ludlum and Magee (9) and Gerchman and Ludlum (10) have reported that transcriptional errors were made by RNA polymerase *in vitro* with templates containing 3-methylcytosine, 3-ethylcytosine, or O^6-methylguanine but not with 7-methylguanine (13), one of the major products of DNA alkylation by MNU. A number of investigators have looked for O^6-alkylguanine in nucleic acids alkylated by various agents *in vivo* and *in vitro* and have established that the relative amount of O^6-alkylguanine appeared to correlate with the reported carcinogenicity or mutagenicity of the various alkylating agents [summarized by Singer (14)].

Masters and Pardee (15) have shown that the function of proteins synthesized after exposure to ultraviolet light is a more sensitive indicator of DNA damage than other parameters such as total synthesis of DNA, RNA, or protein. Accordingly, Chen *et al.* (16) treated DNA from a temperate transducing bacteriophage carrying the *Escherichia coli lac* operon with a methylating agent, dimethyl sulfate, in order to study the relationship between DNA alkylation and the fidelity of DNA template function. Use of this methylated DNA as template for βG synthesis *in vitro* resulted in a decrease in the expected level of newly synthesized βG activity. In this paper we describe our investigation of the effects of the homologous methylating/ethylating agents MNU and ENU on template DNA and its subsequent ability to direct the synthesis of βG *in vitro*. In addition, the relative importance of carbamoylation and of methylation in the inactivation of template DNA by MNU is evaluated.

[1] Abbreviations used: MNU, N-methyl-*N*-nitrosourea; ENU, *N*-ethyl-*N*-nitrosourea; βG, β-galactosidase; S-30, supernatant from centrifugation at 30,000*g*.

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MATERIALS AND METHODS

In Vitro Synthesis and Assay of βG

The *in vitro* DNA-directed system for synthesis of βG and βG assay conditions were described in detail by Chen *et al.* (16). Briefly, template DNA from a transducing, temperate bacteriophage, λh80dlacp^s, which contains the entire *lac* operon from *E. coli*, is transcribed to messenger RNA which in turn is translated to functional protein, including β-galactosidase, by enzymes and ribosomes present in a 30,000*g* supernatant prepared from a *lac*-deleted strain of *E. coli*. Newly synthesized β-galactosidase is quantitated by a colorimetric method based on cleavage of a colorless substrate, *o*-nitrophenyl-β-D-galactopyranoside, to form yellow *o*-nitrophenol.

All reagents were obtained from the same sources as before (16), with the following exceptions: β-mercaptoethanol was purchased from Eastman Organic Chemicals, methylamine hydrochloride and potassium cyanate (KNCO) from Fisher Scientific Company, sodium nitrite from Mallinckrodt Chemical Company, and ppGpp from PL Biochemicals. The complete system (70 μl) contained S-30 (480 μg protein), λh80dlacp^s DNA (2 μg, with or without pretreatment with MNU or ENU), salts, and cofactors. βG activity from DNA exposed to MNU or ENU was expressed as a percentage of the control value obtained using the same quantity of untreated DNA.

Chemicals

MNU (Ash Stevens Inc.) and ENU (prepared in this laboratory by nitrosation of ethylurea as in Ref. 17) were dissolved in pH 6.0 deionized H_2O, divided into small portions, and stored at −20°C. Each portion was used immediately after thawing.

Radiolabeled Chemicals

^{14}C-Potassium cyanate (48.4 mCi/mmol), methyl-^{3}H-MNU (1.055 Ci/mmol), methyl-^{14}C-MNU (13.3 mCi/mmol), and ethyl-1-^{14}C-ENU (11.5 mCi/mmol) were purchased from New England Nuclear Corp. and diluted with cold materials to the specific activities described in each experiment. Aquasol (New England Nuclear) was acidified by adding 7.5 ml of acetic acid to 1 liter of the commercial preparation.

Carbonyl-^{14}C-MNU was synthesized by a modification of the method of Lawley and Shah (18). ^{14}C-Potassium cyanate (48.4 mCi/mmol, 1.7 mg) was diluted with an unlabeled aqueous KNCO solution (1.2 mmol in 1.7 ml of H_2O). Methylamine hydrochloride (2.5 mmol) and 1 drop of 0.1 M H_2SO_4 were added to the mixture. The solution was refluxed for 12 min and cooled in ice. Sulfuric acid (2.75 M, 1.2 ml) followed by $NaNO_2$ (2.68 mmol) were added gradually over a period of 25 min. Some carbonyl-^{14}C-MNU precipitated at this point. The whole mixture was extracted with eight 2-ml portions of ether. The ether extracts were combined, washed with H_2O, and dried over anhydrous $MgSO_4$ in a desiccator in the dark for about 1 h. The ether solution was then decanted and evaporated in a desiccator again in the dark, leaving a residue of crystals of carbonyl-^{14}C-MNU (48.96 mg, 0.475 mmol; 46% yield; mp 113–115°C (dec); sp act 0.49 mCi/mmol).

Bacteriophage λh80dlacp^s DNA and S-30 Extract from E. coli

Bacteriophage λh80dlacp^s DNA and S-30 from *E. coli* W4032 cells were prepared as described by Chen *et al.* (16). The protein content of S-30 was determined by Bio-Rad protein assay (19) with crystalline bovine serum albumin as the reference standard.

DNA Treatment with Alkylnitrosourea

λh80dlacp^s DNA (238 μg/ml) was exposed to 0.73–12.9 mM MNU or 6.24–50 mM ENU in 5 mM Tris–Cl buffer, pH 8.0. After 10 min at 37°C, the reaction mixture was cooled to 0°C with ice and the unreacted MNU or ENU was removed by dialysis at 4°C versus 10 mM Tris–Cl, pH 8.0. Control DNA solutions were treated in the same fashion but without nitrosourea. The final concentration of the treated DNA was determined by measuring absorbance at 260 nm. Fifty microliters of the treated DNA solution was mixed with 15 ml of acidified aquasol solution and counted in a Beckman LS-8100 scintillation counter. The extent of DNA alkylation or carbamoylation was calculated from the specific activities of labeled MNU or ENU, the cpm in 50 μl treated DNA, and the treated DNA concentration according to the following equation: Percentage DNA nucleotides alkylated (or carbamoylated) = [(cpm treated DNA − cpm control DNA) × 100]/[(specific activity of alkylnitrosourea, cpm/mol) × (mol nucleotides in 50 μl DNA solution)].

MNU and ENU Effects on βG Synthesis in Vitro

In situ reaction. Various concentrations of MNU or ENU solutions were added to the complete system including DNA at the beginning of incubation. After 60 min βG activity was determined.

Delayed addition effect. MNU or ENU solution (5 μl) was added to the complete system (70 μl) at various times after incubation had begun. The total incubation time was 70 min. βG activities under different conditions were measured as a percentage of the control.

DNA inactivation by alkylnitrosourea followed by immediate use as template. DNA was incubated with MNU or ENU for up to 10 min at 37°C. S-30, salts, and cofactors were then added to complete the system, and incubation was continued for 60 min before βG activity was assayed.

S-30 inactivation by alkylnitrosourea in situ. S-30 and MNU or ENU were preincubated for various times at 37°C. DNA, salts, and cofactors were then added. The final concentrations of each component were the same as in the complete system. The mixture was incubated for another 60 min, and βG activities were determined.

Effect of KNCO on βG Synthesis

In order to study the effects of carbamoylation on this system, a pure carbamoylating agent, KNCO, was used instead of alkylnitrosourea both to treat DNA and in the *in situ* inhibition reaction. DNA (400 μg/ml) was exposed to 12.5 mM KNCO in 5 mM Tris–Cl buffer, pH 8.0, for 10 min at 37°C. After removal of the unreacted KNCO by dialysis, treated DNA was tested for template activity. For *in situ* inhibition, KNCO at concentrations of up to

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14 mM was added to the complete system. After incubation for 60 min, βG activity was assayed. For comparison, simultaneous control reactions were carried out with MNU and ENU.

RESULTS

DNA Alkylation by MNU and ENU

The extent of DNA alkylation after 10 min at 37°C increased with the concentration of the alkylating agent (Fig. 1) and, after reaction with either MNU or ENU, was linear in the range of 0–1% nucleotide alkylation. At equal concentrations, MNU was more reactive than ENU toward DNA with respect to both rate and extent of alkylation. Methylation was 12.5 times as extensive as ethylation after 10 min of reaction at 37°C and pH 8.0.

DNA Carbamoylation by MNU

Serebryanyi *et al.* have demonstrated that both carbamoylation and methylation occurred in animal DNA treated with MNU *in vitro* (3). Under our experimental conditions, carbamoylation of phage DNA by MNU was a very minor reaction (Table 1). Thirteen millimolar MNU yielded less than 0.03% carbamoylated nucleotides; under the same conditions, 1.5% of the DNA nucleotides were methylated.

Effect of MNU and ENU on DNA Template Function

When MNU- or ENU-pretreated DNA was used as template for synthesis of βG in vitro, βG activities decreased as MNU or ENU concentrations increased (Fig. 2). There was no detectable isotope effect on the rate of reaction when radiolabeled compounds were used. Although the effect of a given level of exposure to MNU appeared greater than that of comparable exposure to ENU, this conclusion was modified by analysis of the extent of alkylation as a function of nitrosourea concentration. βG activity was plotted against the percentage nucleotide alkylation of template DNA by both alkylnitrosoureas (Fig. 3). A given percentage of nucleotide alkylation, either methyl or ethyl, resulted in the same degree of inhibition of βG activity. These experiments were repeated with different preparations of MNU or ENU having different specific activities, and comparable results were obtained.

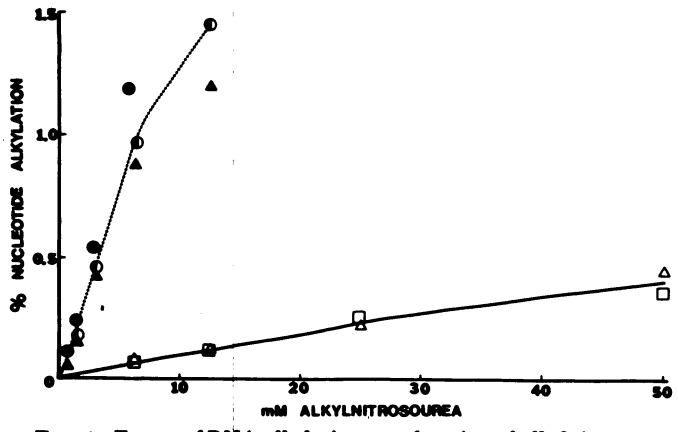

FIG. 1. *Extent of DNA alkylation as a function of alkylnitrosourea concentration after incubation at 37°C for 10 min*

Solid symbols indicate MNU; open symbols, ENU. Specific activities are: methyl-^{3}H-MNU, 0.691 mCi/mmol (●); methyl-^{14}C-MNU, 0.645 mCi/mmol (◐); methyl-^{14}C-MNU, 0.605 mCi/mmol (▲); ethyl-1-^{14}C-ENU, 5.18 mCi/mmol (△); ethyl-1-^{14}C-ENU, 1.78 mCi/mmol (□), respectively.

TABLE 1

Dependence of DNA carbamoylation and methylation on MNU concentration

λh80dlacp[a] DNA was incubated with various concentrations of MNU at 37°C for 10 min, then isolated and assayed as described in Materials and Methods.

DNA carbamoylation			DNA methylation		
mM MNU[a]	% Carbamoylation	% βG activity[b]	mM MNU[c]	% Alkylation	% βG activity[b]
0	0	100	0	0	100
1.61	0.01	81	1.56	0.18	72
3.22	0.01	58	3.12	0.45	57
6.45	0.01	38	6.25	0.97	29
12.9	0.03	31	12.5	1.46	12

[a] Carbonyl-^{14}C-MNU, sp act 0.47 mCi/mmol.
[b] Each value represents the mean of two experiments.
[c] Methyl-^{14}C-MNU, sp act 0.645 mCi/mmol.

Effect of DNA Carbamoylation by KNCO on βG Synthesis

Pretreatment of DNA with up to 12.5 mM KNCO had no effect on synthesis of βG activity (100 ± 5.4% of the control value). Simultaneous control pretreatment of aliquots of DNA with 12.5 mM MNU, as expected, de-

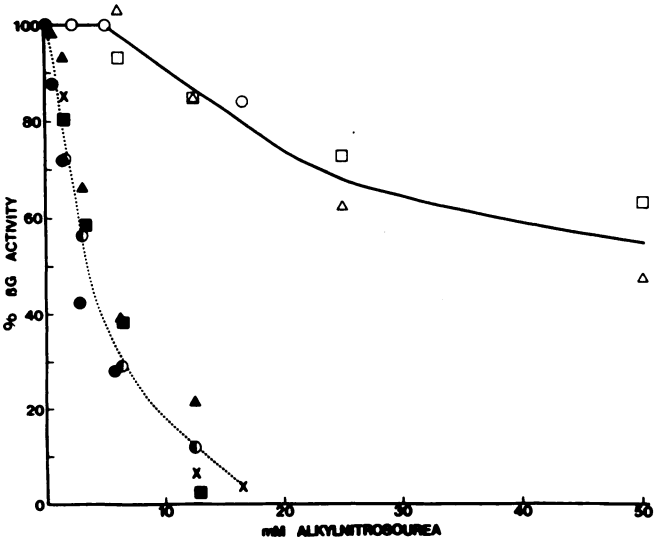

FIG. 2. *Template activity of λh80dlacp[a] DNA for β-galactosidase synthesis after incubation with MNU or ENU at the concentrations indicated*

DNA was treated with alkylnitrosourea at 37°C for 10 min, then dialyzed at 4°C against 10 mM Tris–Cl, pH 8.0. Pretreated dialyzed DNA was used as template in the complete β-galactosidase synthesis system. Solid symbols represent MNU; open symbols, ENU. Each point indicates the mean value from two experiments. Specific activities for each treatment were: methyl-^{3}H-MNU, 0.691 mCi/mmol (●); methyl-^{14}C-MNU, 0.645 mCi/mmol (◐); methyl-^{14}C-MNU, 0.605 mCi/mmol (▲); CO-^{14}C-MNU, 0.47 mCi/mmol (■); ethyl-1-^{14}C-ENU, 5.18 mCi/mmol (△); ethyl-1-^{14}C-ENU, 1.78 mCi/mol (□); cold MNU (X); cold ENU (○).

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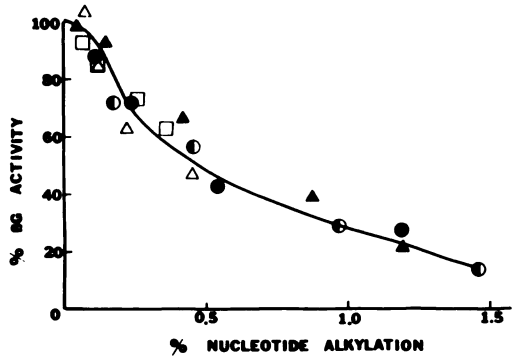

FIG. 3. *Relationship between DNA alkylation and DNA template activities*

Solid symbols indicate MNU treatment; open symbols, ENU. Each point indicates the mean result from two experiments. Different symbols represent MNU and ENU samples of varying specific activities as in Figs. 1 and 2.

creased βG activity to 8 ± 4% of the control values. Since MNU and KNCO have equivalent carbamoylating activity *in vitro* (20), our results indicate that DNA alkylation rather than carbamoylation was responsible for inhibition of DNA template activity by MNU.

Effects of MNU and ENU on the βG Synthetic Mixture Including DNA

In situ reaction. When MNU or ENU was added to the complete βG system at the beginning of incubation, formation of βG activity was inhibited in proportion to the concentration of nitrosourea (Table 5). Under the same conditions, MNU was more inhibitory than ENU.

Effect of delayed addition of MNU or ENU. When MNU or ENU was added to the complete system at various times after the beginning of incubation, the extent of inhibition of βG synthesis decreased. The later the addition, the less inhibition was observed (Table 2). This delayed addition effect depended upon the molar ratio (R) of alkylnitrosourea to DNA nucleotides; higher concentrations of nitrosourea could affect inhibition after a longer delay than lower concentrations. For example, with MNU and R=17, there was no inhibition of βG activity when MNU was added 10 min after incubation had started, but when R=34, inhibition occurred until 20 min after inception. With ENU, when R=34 no inhibition was observed if addition took place after 10 min; twice the corresponding R value was necessary to prolong the period of potential inhibition to 20 min. This clearly indicated that MNU and ENU were most inhibitory during the early stages of the coupled transcription/translation βG synthesis system and further demonstrated the greater potency of MNU as an inhibitor.

Effects of MNU and ENU on DNA or S-30 in Situ

In order to study whether the template DNA or S-30 was the more important target for MNU and ENU as measured by inhibition of βG synthesis, either DNA or S-30 was preincubated with MNU (or ENU). The other components required for the complete system were then added, incubation was continued for 60 min, and βG activities were assayed.

DNA inactivation by MNU or ENU in situ. This, followed by βG synthesis without removal of residual nitrosourea by dialysis, is shown in Table 3. With MNU, inhibition of βG activity increased as the preincubation interval with DNA increased, especially during the first 5 min. But this time-dependent inhibitory effect disappeared when buffer or the salts and cofactors were present in the preincubation mixture, possibly due to accelerated decay of MNU under these conditions (21). With ENU, inhibition increased very slightly as the preincubation time increased.

Inactivation of S-30 in situ. This was studied by incubating MNU or ENU with S-30 for various periods of time up to 30 min, after which DNA, salts, and cofactors were added and βG activity was determined after the further elapse of 1 h. Table 4 illustrates the results of such experiments. Significant depression of βG activity was seen when the period of incubation with S-30 was twice the half-life of MNU under these conditions; the extent of inhibition was comparable to that seen when either ENU or MNU was added to the complete system at the beginning of βG synthesis, and is not attributable to reaction of the nitrosoureas solely with DNA. The compounds themselves or their degradation products, such as isocyanate anion, apparently were reactive with non-DNA components of the mixture.

TABLE 2

Effect of delayed addition of MNU or ENU on the complete β-galactosidase synthesis system

MNU (3.5 mM) or ENU (7 mM) was added to the complete protein synthesis system at various times (t) after the beginning of incubation. Total incubation time was 60 min.

MNU				ENU			
DNA nucleotide conc.	Molar ratio, MNU:nucleotide	t	% βG activity remaining	DNA nucleotide conc.	Molar ratio, ENU:nucleotide	t	% βG activity remaining
mM		*min*		*mM*		*min*	
0.103	34	0	41 ± 7	0.103	68	0	43
		2	43 ± 4			2	49 ± 3
		6	60 ± 5			6	75 ± 3
		10	75 ± 5			10	74 ± 3
		20	101 ± 5			20	101 ± 5
0.206	17	0	30 ± 1	0.206	34	0	43 ± 8
		2	31 ± 1			2	43 ± 9
		5	56 ± 6			5	55 ± 7
		10	99 ± 8			10	92 ± 8

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TABLE 3

DNA inactivation by MNU or ENU in situ

λh80dlacp[a] DNA (2.36 μg, 5 μl) was preincubated with 5 μl MNU or ENU solution for various times (*t*) before time 0, when the other components were added to complete the βG synthesizing system. Incubation was then continued for another 60 min before βG activity was assayed.

MNU			ENU		
mM MNU[a]	*t*	% βG activity remaining	mM ENU[a]	*t*	% βG activity remaining
	min			*min*	
1.56	0	101 ± 1	2.08	1	92 ± 3
	5	47 ± 10		3	82
	10	42 ± 11		5	91 ± 5
				10	83 ± 2
3.12	0	79	4.16	1	97 ± 4
	5	20 ± 1		3	86
	10	13 ± 1		5	89 ± 8
				10	88 ± 3
6.25	0	66 ± 5	8.33	1	85 ± 3
	5	17 ± 4		3	73 ± 4
	10	12 ± 2		5	75 ± 4
				10	71 ± 3

[a] Concentration in preincubation mixture.

TABLE 4

S-30 inactivation by MNU or ENU in situ

S-30 (20 μl, 17.1 mg/ml) was incubated with 5 μl of MNU or ENU for various times (*t*) before time 0, when the remaining components were added to complete the βG synthesizing system. Incubation was then continued for an additional 60 min, after which the mixture was assayed for βG activity.

MNU			ENU		
mM MNU[a]	*t*	% βG activity remaining	mM ENU[a]	*t*	% βG activity remaining
	min			*min*	
3.57	0	51 ± 1	7.14	0	56 ± 1
	10	43 ± 1		10	47
	30	51 ± 1		30	48
7.14	0	22 ± 5	14.3	0	30 ± 1
	10	26 ± 1		10	25 ± 1
	30	23 ± 3		30	22 ± 1

[a] Concentration during initial incubation.

Effect of KNCO on the βG Synthetic Mixture

When KNCO was added to the complete βG synthesis system at the beginning of incubation, synthesis of βG activity was inhibited (Table 5). Inhibition was proportional to the concentration of KNCO. *In situ* inhibition reactions with MNU and ENU were performed as simultaneous controls to allow direct comparison of the effects of pure carbamoylation and of combined carbamoylation and alkylation. Since exposure of DNA to KNCO ≤ 12.5 mM had no effect on its ability to direct synthesis of βG activity, we attribute the *in situ* KNCO inhibition effect shown in Table 5 to consequences of carbamoylation of non-DNA components of the synthetic mixture.

DISCUSSION

Alkylnitrosoureas are chemically reactive, alkali-labile compounds, which decompose under physiological conditions to yield an alkylating moiety, formally an alkyldiazonium cation, and isocyanate, an anion capable of carbamoylation of nucleophilic groups including amino and thiol functions. Although many investigators have emphasized the important role of the alkylating activity of MNU and its homologs in mutagenicity and tumorigenicity (reviewed in Ref. 6), both carbamoylation and alkylation have been shown to occur in DNA exposed to MNU *in vitro*, and carbamoylation has been proposed as a potential mutagenic reaction (3). Our finding that carbamoylation of phage DNA by MNU was a very minor reaction (Table 1) and that treatment of DNA with KNCO had no effect on its template function for βG synthesis confirms that in this system, alkylation is the only significant class of MNU reactions with DNA insofar as template function is concerned. However, the inhibition of the complete βG synthesis system by KNCO suggests that carbamoylation of non-DNA components of the protein synthetic machinery constitutes a potentially important toxic, if not mutagenic, reaction. This is in accord with the observations of Knox (22) that carbamoylation of serum components is responsible for the cytotoxicity of MNU and ENU in cell culture. Therefore, the inhibition of βG synthesis when MNU or ENU is added to the complete system is the combined effect of alkylation of DNA and carbamoylation of S-30 components.

Lawley *et al.* (12) and Singer and co-workers (14, 23) demonstrated that DNA exposed to MNU or ENU at pH 7.8 contained different distributions of alkylated bases and that, for a given agent, similar distributions of alkylated bases resulted from reaction with DNA *in vivo* or *in vitro*. It is reasonable to postulate that the observations of these workers also apply to the reactions of MNU and ENU with phage DNA in our studies. Thus, although specific alkylation products were not isolated and quantitated in our studies, we assume that for a given level of total alkylation, the ratio (O^6-alkylguanine/7-alkylguanine) was much greater in DNA exposed to ENU than in MNU-treated DNA. This is important in view of the findings by many investigators that the carcinogenicity or mutagenicity of various alkylating agents is directly proportional to the relative amount of O^6-alkylguanine among the DNA alkylation products (14). Our finding that synthesis of βG activity depended on the total extent of DNA alkylation, irrespective of whether methylation or ethylation had occurred (Fig. 3), indicates that O^6-

TABLE 5

In situ inhibition of β-galactosidase synthesis by KNCO, MNU, and ENU

Details given in Materials and Methods. Each value represents the mean of at least two experiments.

mM	% βG Activity		
	KNCO	MNU	ENU
0	100	100	100
1.75	85 ± 4	63 ± 2	85 ± 1
3.5	75 ± 11	48 ± 4	72 ± 4
7.0	47 ± 7	9 ± 7	28

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alkylguanine is unlikely to have been the only significant lesion capable of inhibiting DNA template function. This result is in accord with the results of Mamet-Bratley, who found that the inhibition of phage T-7 DNA as a template for RNA synthesis by *E. coli* RNA polymerase resulting from preexposure of the DNA to methyl or ethyl methanesulfonate was directly proportional to the level of total DNA alkylation and independent of the nature (and presumably the sites of reaction) of the alkyl groups (24).

It is clear from the results of DNA inactivation and S-30 inactivation *in situ* (Tables 3 and 4) that DNA was an important target for MNU, but that it was not the only significant target; that DNA and components of the S-30 mixture compete as reactants for the nitrosourea; and that reaction of MNU with either can inhibit βG synthesis. The rate of reaction of MNU with DNA was extremely rapid in comparison with its next higher homologue, ENU. However, in agreement with previous studies (25), the addition of either compound to a βG reaction mixture was progressively less inhibitory as more time elapsed between the inception of transcription/translation and the addition of the alkylnitrosourea. Kung *et al.* (26) showed that in this system maximal mRNA initiation occurs during the first 6 min, while βG activity continues to increase for 60 min or more. Early stages of the coupled transcription/translation system thus appear most sensitive to inhibition by both alkylnitrosoureas, which is consistent with the primary importance of DNA as a target for both agents even in the presence of competing target substances, such as RNA, which are also targets for electrophilic reactants and which can be inactivated by such reactions (27, 28).

The exact mechanism by which alkylating agents including MNU and ENU affect DNA template function remains to be clarified. Among the mechanisms that can be envisioned are the following: (a) Alkylated bases in DNA may result in miscoding mRNA in the transcriptional stage, resulting in turn in the synthesis of altered protein or no protein by miscoding in the translation stage; (b) loss of negative charge on DNA by the formation of phosphotriester may affect the binding and movement of enzymes on the DNA (8) which in turn might affect the synthesis of mRNA; and (c) DNA strand breakage subsequent to depurination (29) or phosphotriester formation (30) may radically inhibit DNA template activity. Further investigations of the effects of alkylating agents in this system are in progress.

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