Reaction of Nitrosoureas with Polycytidylate Templates for Ribonucleic Acid Polymerase

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(Received 19 April 1972)

Many chemical mutagens and carcinogens have been shown to react with nucleic acids *in vivo*, adding substituent groups to nucleophilic sites on the nucleic acid bases (Miller & Miller, 1966). Although such reactions have not been directly related to carcinogenesis, some relationship between the two phenomena may exist. This relationship could result, for example, from an alteration in the properties of the substituted nucleic acids when they act as templates for either DNA or RNA synthesis.

Alterations in nucleic acid templates produced by classical alkylating agents have been studied with synthetic polynucleotides of known composition (Ludlum & Wilhelm, 1968; Ludlum, 1970*a,b*). These templates provide information for the synthesis of a new strand of RNA polymer when they are incubated with RNA polymerase and suitable substrates. Changes produced by alkylation can be detected rather easily as differences in the composition of the product polymer.

If ultimate carcinogens and mutagens produce their effects by reacting with nucleic acids, their activity might be reflected in their ability to change the template properties of synthetic polynucleotides (Troll *et al.*, 1968; Singer & Fraenkel-Conrat, 1970). The RNA polymerase system could then be used to study the activity of known or suspected ultimate carcinogens, and would also distinguish between those compounds that have a direct biological action and those that must be metabolized before they are active.

The *N*-nitroso compounds include some of the most powerful mutagenic and carcinogenic chemicals known. Most, but not all, of the experimental evidence indicates that the biological activity of these compounds is mediated by products of their decomposition rather than by the unchanged parent molecules (Druckrey *et al.*, 1967; Magee & Barnes, 1967; Magee & Swan, 1969). Some nitrosamines, such as dimethylnitrosamine, can behave as alkylating agents *in vivo* after enzymic decomposition, whereas nitrosamides, such as *N*-methyl-*N*-nitrosourea, decompose spontaneously under physiological conditions to form alkylating agents. However, it is not known whether the biological behaviour of the nitroso

compounds can be explained by their capacity to alkylate cellular components, and other possible biologically active intermediates have been suggested. These include the corresponding hydrazine derivative (Suss, 1965), the hydroxamic acid (Neunhoeffer *et al.*, 1970) and nitrous acid (Zimmermann *et al.*, 1965; Craddock, 1969). Further, there are differences in the biological activities of the alkylating agents methyl methanesulphonate and ethyl methanesulphonate and the corresponding nitrosamides (Loveless & Hampton, 1969; Swann & Magee, 1969). It was therefore decided to compare the effects of some biologically active nitroso compounds on the polynucleotide templates with those previously obtained with alkyl methanesulphonates (Ludlum & Wilhelm, 1968; Ludlum, 1970*a,b*).

In the experiments described below we have modified poly(C) by reaction with *N*-methyl-*N*-nitrosourea and with *N*-ethyl-*N*-nitrosourea, both of which have very potent mutagenic and carcinogenic activity. Poly(C), which stimulates poly(G) formation with RNA polymerase, is known to cause misincorporation of UMP after it has reacted with methyl methanesulphonate (Ludlum & Wilhelm, 1968) or with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Singer & Fraenkel-Conrat, 1970).

The sample of poly(C) used in these experiments was obtained from the Miles Chemical Co. (Elkhart, Ind., U.S.A.). *N*-Methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea were synthesized by Dr. P. F. Swann by previously described methods (Swann & Magee, 1968, 1971). Other reagents were obtained from standard commercial sources. Substituted polymers were produced by reaction of poly(C) with the appropriate nitrosourea. In the experiments reported here, 0.3 ml of poly(C) solution (10 mg/ml in 0.2 M-sodium cacodylate buffer, pH 7) was mixed with 3 mg of the dry nitrosourea, and the tubes were incubated overnight at 37°C. Dissolution of methyl-nitrosourea and ethylnitrosourea took place slowly over the first hour and was aided by occasional shaking. After incubation the substituted polymers were precipitated with an excess of ethanol, redissolved in cacodylate buffer and reprecipitated. Finally they were dissolved in 0.1 M-tris-HCl buffer,

pH 7.5, and used as stock solutions in the RNA polymerase reactions. Concentrations were determined from the absorbance by using the extinction coefficient for poly(C) given by Warner (1957). A portion of each product was hydrolysed with 1M-HCl at 100°C for 30 min. The hydrolysates were then neutralized and applied to a 0.5 cm × 5.5 cm column of Dowex AG-1 (X2; 200–400 mesh) followed by elution with an exponential gradient of lithium acetate in 0.01M-ammonium acetate buffer, pH 6.8. Insufficient derivative was obtained for positive identification, but u.v.-absorbing peaks were obtained in the regions corresponding to 3-methyl-CMP from the hydrolysate of methylnitrosourea-treated poly(C) and to 3-ethyl-CMP from the hydrolysate of ethylnitrosourea-treated poly(C). A substi-

tution of 10.5% was obtained for the methylnitrosourea-treated poly(C) and of 3.6% for the ethylnitrosourea-treated poly(C), calculated as described by Ludlum (1970b).

RNA polymerase was isolated from *Micrococcus luteus* by the procedure of Nakamoto *et al.* (1964). Incubations were performed with enzyme and polynucleotide templates for 1 h at 30°C in the presence of substrate, including one ¹⁴C-labelled nucleoside triphosphate, Mn²⁺ and buffer as described by Ludlum (1970b).

RNA synthesis directed by alkyl nitrosourea-treated templates is shown in Figs. 1 and 2. Since even the modified polymers consist primarily of CMP units, the main component of the newly synthesized RNA strand is GMP. Fig. 1, showing the incorporation of GMP, demonstrated that template activity is very seriously affected by reaction with the carcinogenic nitrosoureas. Incorporation by methylnitrosourea-treated poly(C), for example, is 10% or less of the incorporation by the unmodified poly(C).

A very significant misincorporation of UMP

Fig. 1. GMP incorporation in the presence of methylnitrosourea-treated and ethylnitrosourea-treated poly(C)

Reaction mixtures (final volume 0.125 ml) contained (final concentrations): tris-HCl buffer, pH 7.5, 0.1 M; MnCl₂, 2.5 mM; [¹⁴C]GTP (0.21 μCi/μmol), 1.2 mM; UTP, 1.2 mM; enzyme, 35 μg; template [○, poly(C); ●, methylnitrosourea-treated poly(C); ◐, ethylnitrosourea-treated poly(C)], as indicated. Incubation was for 60 min at 30°C. Assay was as described in the text.

Fig. 2. UMP incorporation in the presence of methylnitrosourea-treated and ethylnitrosourea-treated poly(C)

Reaction mixtures (final volume 0.125 ml) contained (final concentrations): tris-HCl buffer, pH 7.5, 0.1 M; MnCl₂, 2.5 mM; [¹⁴C]UTP (0.21 μCi/μmol), 1.2 mM; GTP, 1.2 mM; enzyme, 35 μg; template [○, poly(C); ●, methylnitrosourea-treated poly(C); ◐, ethylnitrosourea-treated poly(C)], as indicated. Incubation was for 60 min at 30°C. Assay was as described in the text.

occurs when the modified polymers are incubated with RNA polymerase (Fig. 2). If the percentage of UMP in the product is calculated from the combined data of Figs. 1 and 2, a correspondence with the percentage of substitution in the template [3.6% for ethylnitrosourea-treated poly(C) and 10.5% for methylnitrosourea-treated poly(C)] emerges. Thus substitution of cytosine by reaction with these carcinogens evidently caused a misreading of the template by RNA polymerase. Control experiments, on the other hand, demonstrated that no misincorporation occurred when the templates were incubated with methylurea or ethylurea, compounds that have not been reported to be carcinogenic.

Since the dialkylnitrosamines may require prior metabolic changes to achieve biological activity, it was decided to test their effects on the template. Replicate samples of poly(C) at a concentration of 10mg/ml were incubated with dimethylnitrosamine (0.14M) for 16h at 37°C in cacodylate buffer, pH7, and the effect on UMP incorporation was compared with that of incubation of the polymer without the nitrosamine; additional samples were incubated with methylnitrosourea as positive controls. The mean UMP incorporation with four samples of poly(C) incubated with dimethylnitrosamine was 0.0029 ± 0.0007 nmol of UMP/nmol of template, which was probably not significantly different from that with five samples of template incubated alone, namely 0.0018 ± 0.0011 nmol/nmol ($0.1 > P > 0.05$). The mean incorporation after incubation of eight samples of poly(C) with methylnitrosourea (0.10M) was 0.03 ± 0.0118 nmol/nmol, representing a highly significant increase above the controls ($P < 0.001$). Duplicate incubations of poly(C) with diethylnitrosamine (0.10M) and *N*-nitrosomorpholine (0.09M) gave no indication of a decrease in GMP incorporation. These results are consistent with a requirement for metabolic activation of the dialkylnitrosamines.

We have therefore demonstrated a direct effect of certain carcinogens on the biochemical properties of

nucleic acid templates. Further experiments will be required to determine whether or not this effect is a general one for ultimate carcinogens of different chemical types.

This work was briefly reported at the Symposium of the Princess Takamatsu Cancer Research Fund, Tokyo, Japan, held on 16–18 November 1971. The skilled technical assistance of Mr. John Holsman and Mr. Raymond Mace is gratefully acknowledged. This work was supported by Grant T-432 from the American Cancer Society, by U.S. Public Health Service Grant GM-16952 and by the Cancer Research Campaign of Great Britain. D. B. L. is a Markle Scholar in Academic Medicine at the University of Maryland School of Medicine.

- Craddock, V. M. (1969) *Biochem. J.* **111**, 615
 Druckrey, H., Preussmann, R., Ivankovic, S. & Schmahl, D. (1967) *Z. Krebsforsch.* **69**, 103
 Loveless, A. & Hampton, C. L. (1969) *Mutat. Res.* **7**, 1
 Ludlum, D. B. (1970a) *J. Biol. Chem.* **245**, 477
 Ludlum, D. B. (1970b) *Biochim. Biophys. Acta* **213**, 142
 Ludlum, D. B. & Wilhelm, R. C. (1968) *J. Biol. Chem.* **243**, 2750
 Magee, P. N. & Barnes, J. M. (1967) *Advan. Cancer Res.* **10**, 163
 Magee, P. N. & Swann, P. F. (1969) *Brit. Med. Bull.* **25**, 240
 Miller, E. C. & Miller, J. A. (1966) *Pharmacol. Rev.* **18**, 805
 Nakamoto, T., Fox, C. F. & Weiss, S. B. (1964) *J. Biol. Chem.* **239**, 167
 Neunhoffer, O., Wilhelm, G. & Lehmann, G. (1970) *Z. Krebsforsch.* **256**, 302
 Singer, B. & Fraenkel-Conrat, H. (1970) *Biochemistry* **9**, 3694
 Suss, R. (1965) *Z. Krebsforsch.* **206**, 714
 Swann, P. F. & Magee, P. N. (1968) *Biochem. J.* **110**, 39
 Swann, P. F. & Magee, P. N. (1969) *Nature (London)* **223**, 947
 Swann, P. F. & Magee, P. N. (1971) *Biochem. J.* **125**, 841
 Troll, W., Belman, S., Berkowitz, E., Chmielewicz, Z. F., Ambrus, J. L. & Bardos, T. J. (1968) *Biochim. Biophys. Acta* **157**, 16
 Warner, R. C. (1957) *J. Biol. Chem.* **229**, 711
 Zimmermann, F. K., Schwaier, R. & von Laer, U. (1965) *Z. Vererbungslehre* **97**, 68