Cross-linking of DNA induced by chloroethylnitrosourea is prevented by O\textit{\textsubscript{6}}-methylguanine-DNA methyltransferase

Peter Robins, Adrian L. Harris*, Iain Goldsmith and Tomas Lindahl

Imperial Cancer Research Fund, Mill Hill Laboratories, London NW7 1AD, UK

Received 26 September 1983; Accepted 21 October 1983

ABSTRACT

The DNA repair enzyme O\textit{\textsubscript{6}}-methylguanine-DNA methyltransferase has been used as a reagent to analyse the initial reaction sites of alkylating agents such as chloroethylnitrosourea that cross-link DNA. The transferase can be employed for this purpose because it removes substituted ethyl groups from DNA, as shown by its ability to act on O\textit{\textsubscript{6}}-hydroxyethylguanine residues in DNA. The enzyme counteracts the formation of interstrand cross-links induced by bis-chloroethylnitrosourea, but not those induced by nitrogen mustard. Once formed, chloroethyl-nitrosourea-induced cross-links are not broken by the enzyme. In agreement with deductions from experiments with living cells, it is concluded that chloroethylnitrosoureas act by forming reactive monoadducts at the O\textit{\textsubscript{6}} position of guanine and/or the O\textit{\textsubscript{4}} position of thymine, which subsequently generate -CH\textsubscript{2}CH\textsubscript{2}- bridges to the complementary DNA strand. A new method for quantitating interstrand cross-links in DNA has been employed.

INTRODUCTION

Cross-linking alkylating agents such as nitrogen mustards and halogenated ethylnitrosoureas are strongly cytotoxic, and are clinically useful as chemotherapeutic agents (1). The prevention of DNA strand separation during replication by interstrand cross-links is presumably important for the cell-killing effect, since related alkylating agents without cross-linking ability, such as half-mustards or N-methylnitrosourea, exhibit similar mutagenic effects but are less toxic. A major site of reaction with alkylating agents is the N\textit{\textsubscript{7}} position of guanine. Nitrogen mustards, which are bifunctional alkylating agents, apparently generate DNA interstrand cross-links between guanine N\textit{\textsubscript{7}} sites at GpC sequences (2,3). In contrast, the structure of the DNA cross-links formed by chloroethylnitrosoureas has been less well defined. The latter agents decompose in aqueous solution to generate reactive chloroethyl carbonium ions, which act initially as mono-alkylating agents. However, after alkylation of DNA, the monoadducts are further reactive, and in a slow,
subsequent process, the chlorine is eliminated and a -CH₂CH₂- bridge between nucleosides generated (4-6). A short bridge of this type (in contrast to the longer ones formed by nitrogen mustards) could only be accommodated between closely adjacent positions, such as those found at hydrogen-bonded sites, on two DNA strands unless a major structural distortion of the double-helical structure occurred. For this reason, interstrand cross-links caused by chloroethylnitrosoureas probably do not involve the N⁷ position of guanine. Kohn (5) has instead proposed an initial alkylation event at the 0⁶ position of a guanine residue, followed by cross-link formation to the N⁴ position of its corresponding base-paired cytosine, as a likely possibility. In support of this notion, it has been observed that mammalian cell lines of the Mer⁻ (or Mex⁻) phenotype, which have reduced capacity to repair 0⁶-alkylguanine, show higher levels of chloroethylnitrosourea-induced cross-linking than do normal cells (7). In these experiments, drug-treated cells were lysed on membrane filters, and the amount of interstrand cross-linking was estimated from the rate of alkaline elution of the DNA. By similar methods, it has been found that pretreatment of Mer⁺ cells with a monofunctional alkylating agent causes a temporary conversion to a Mer⁻-like phenotype during subsequent challenge with a cross-linking agent, presumably by saturation of the DNA repair capacity of the cells (8).

These indications from experiments with mammalian cells have not, so far, been directly confirmed by chemical analysis of the cross-linked residues, isolated from purified DNA treated with chloroethylnitrosourea. Two types of cross-linked bases have been detected in such alkylated DNA. One of these, di[7-guanyl]ethane, most likely reflects an intrastrand reaction between two adjacent G residues rather than an interstrand cross-link (9). The other cross-linked structure, however, has an ethyl link between the N³ position of a cytosine and N¹ of guanine, and this presumably represents an interstrand cross-link between previously hydrogen-bonded residues. In order to reconcile these two sets of data, it was proposed that interstrand cross-link formation might have been preceded by initial chloroethylation at the 0⁶ position of guanine, followed by an internal rearrangement involving the N¹ position, prior to cross-link formation with the complementary cytosine residue (10).

The enzyme responsible for removal of alkyl groups from the 0⁶ position of guanine during DNA repair has recently been purified to apparent homogeneity from E.coli (11). Since this activity does not
recognize N-alkylated purine or pyrimidine residues (12; T. McCarthy, P. Karran and T. Lindahl, ms. in preparation), it provides a suitable reagent for investigating the initial site of DNA alkylation by chloro-ethylnitrosoureas. Such experiments are described here.

MATERIALS AND METHODS

Reagents

Bacteriophage M13mp9 were grown in E. coli JM103 (13). Covalently closed circular double-stranded DNA (M13 replicative form) was made from phage-infected bacteria (14) while circular single-stranded DNA was obtained from virus particles (13). Radioactively labeled M13 replicative form DNA (2600 cpm ⋅ µg⁻¹) was obtained by addition of ³H-thymidine (1mCi⋅µl⁻¹) and adenosine (50 µg⋅µl⁻¹) to the culture medium two hours after phage infection. Double-stranded linear M13mp9 DNA was obtained by cleavage with restriction enzyme EcoRI (13). Single-stranded linear M13 DNA was prepared by incubation of circular single-stranded DNA in 0.1 M NaCl, 10 mM sodium citrate (pH 5.0) at 70°C for 4 min, to introduce about one apurinic site per DNA circle, followed by addition of 0.1 volume 2 M NaOH and incubation at 37°C for 4 hr to introduce chain breaks at these sites (15). Micrococcus luteus DNA was purchased from Miles Laboratories, Inc., and further purified as described (12). 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) was obtained from Bristol-Myers Pharmaceuticals, and nitrogen mustard (N-methyl-bis(2-chloroethyl)amine) was purchased from Sigma. 1-(2-Hydroxyethyl)-1-nitrosourea (HNU) (16,17) was a gift from Dr. W. Lijinsky, NCI-Frederick Cancer Research Facility, U.S.A. Stock solutions of BCNU and HNU were made in cold ethanol, stored at -80°C, and used within two days, while nitrogen mustard was dissolved in 0.1 M HCl and stored frozen at -80°C. ⁰⁶-Ethylguanine was prepared as described (18). ⁰⁶-Hydroxyethylguanine and ⁷-hydroxyethylguanine, prepared according to Ashby et al. (19) were gifts from Dr. D. Paton, Imperial Chemical Industries, Ltd. The molar absorption coefficients of these compounds were taken to be the same as those of the corresponding methyl derivatives (20). ⁰⁶-Methylguanine-DNA methyltransferase was the homogeneous E. coli enzyme described by Demple et al. (11). Reduced glutathione, bovine serum albumin (Fraction IV), ethidium bromide, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes), and N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid (Epps) were purchased from Sigma. Albumin was treated with N-ethylmaleimide before use to remove traces of nuclease activity (21).
Proteinase K was purchased from Merck, Seakem agarose LE from Marine Colloids, Ficoll 400 from Pharmacia, and Aquasol from NEN. Sarkosyl was obtained from Ciba-Geigy.

Removal of hydroxyethylated guanine monoadducts from DNA

*M. luteus* DNA was employed in these experiments because of its high (71%) guanine-cytosine content. One ml DNA solution (0.7 mg ml⁻¹) in 200 mM Epps/1 mM EDTA (pH 8.5) was treated with 30 µl of HNU solution (170 mg ml⁻¹, in ethanol). After 90 min at 37°, the reaction mixture was chilled, and the DNA precipitated by the addition of 40 µl 5 M NaCl and 2.2 ml cold ethanol. The DNA was collected on a glass rod, washed extensively in 80% ethanol, redissolved in 0.3 ml of 10 mM Tris-HCl/l mM EDTA (pH 8.0), and dialyzed against the same buffer supplemented with 1 M NaCl for 24 hr at 2°, followed by dialysis against buffer without NaCl for 16 hr.

For determination of alkylated purines, an aliquot of the DNA was precipitated with 1 volume of 0.8 M trichloroacetic acid at 0°. After centrifugation, the precipitate was suspended in 50 µl 0.1 M HCl and incubated at 70° for 30 min. The hydrolysate was centrifuged, and the supernatant analyzed by high pressure liquid chromatography (HPLC) on a Partisil 10-SCX column, employing a gradient from 5 mM to 175 mM ammonium formate (pH 4.0) in 6% methanol. The effluent was monitored in a Perkin-Elmer LS3 spectrofluorimeter equipped with a flow cell, using an excitation wavelength of 286 nm and an emission wavelength of 363 nm. Fluorescence intensities were quantitated by calibration with solutions of alkylated purines of known ultraviolet absorbance.

Enzymatic removal of hydroxyethylguanine residues from DNA employed a similar procedure to that previously described for determining repair of 6-methylguanine (12). The reaction mixture (100 µl) contained 40 µg of HNU-treated DNA in 70 mM Hepes·KOH (pH 7.6)/10 mM dithiothreitol/l mM EDTA, and varying amounts of 6-methylguanine-DNA methyltransferase. After 60 min at 37°, the DNA was acid-precipitated, hydrolyzed, and analyzed by HPLC with fluorescence detection as above.

Measurements of cross-link formation

Reaction mixtures (100 µl in 1.5 ml Eppendorf microtubes) contained double-stranded M13 [³H]DNA (0.8 µg, 2100 cpm) in 70 mM Hepes·KOH (pH 7.6)/1 mM EDTA/0.5 mM dithiothreitol, and either 0.4 µg 6-methylguanine-DNA methyltransferase, 0.4 µg bovine serum albumin, or 0.1 µg reduced glutathione. The tubes were supplemented with BCNU in ethanol (final
concentrations 0.5 mM BCNU, 0.5% ethanol) and incubated at 37° for 3 hr. Control tubes received ethanol without BCNU. In experiments with nitrogen mustard, the final concentration of the alkylating agent was 0.2 mM, incubations were at 30° for 30 min, and reactions were stopped by the addition of 0.02 volume 0.5 M Na$_2$S$_2$O$_3$.

After the alkylation treatment, 2 µl of 10% Sarkosyl and 2 µl of 0.1% proteinase K were added to each reaction mixture, and the incubation was continued for 30 min at 37°. The protease treatment was employed to remove any protein cross-linked to the DNA. The tubes were subsequently chilled to 20°, and the DNA was alkali-denatured by the addition of 100 µl 1 M NaOH. After 3 min, 600 µl of 90% ethanol containing 0.3 M potassium acetate (pH 4.8) were added to neutralize the solution (as checked with pH paper). The tubes were left at -20° for 18 hr to precipitate the samples. Control tubes with native DNA were not alkali-treated but precipitated directly with 3 volumes of 90% ethanol containing 0.3 M potassium acetate (pH 7.5). The DNA precipitates were collected by centrifugation for 15 min in an Eppendorf microcentrifuge, washed in 80% ethanol, and re-centrifuged. The supernatants were removed, and the precipitates each dissolved in 40 µl 10 mM Tris•HCl/1 mM EDTA (pH 8.0). Although the alkylated DNA dissolved less readily than the control DNA, it could be solubilized by keeping the mixture at 0° for 1 hr, and agitating every 15 min by pipetting the sample up and down twenty times in a micropipette tip. Each DNA solution was supplemented with 8 µl of 17.5% Ficoll 400/0.1% bromophenol blue/20 mM Tris•HCl/100 mM EDTA (pH 7.5) and loaded into a well of a 0.8% agarose horizontal slab gel (14.5 x 20 cm) for electrophoresis. The electrophoresis buffer contained 40 mM Tris•acetate/20 mM sodium acetate/18 mM NaCl/2 mM EDTA (pH 8.0), and electrophoretic runs were carried out at 20 V for 24 hr. Gels were stained in ethidium bromide solution (1 mg•l$^{-1}$) for 1 hr, and visualized and photographed in the presence of ultraviolet light. Bands of covalently closed circular DNA (Forms I and IV, see Fig. 3) were excised, transferred to scintillation vials, dissolved by heating for 1 min in 1.5 ml H$_2$O in a microwave oven, and the radioactivities of the samples determined by liquid scintillation counting following addition of Aquasol. For electrophoresis, wells containing DNA not exposed to alkali (lanes 1-3 in Fig. 3) contained 0.4 µg instead of 0.8 µg DNA, to compensate for the approximately two-fold higher fluorescence of Form I DNA compared to the alkali-denatured Form IV DNA after staining with ethidium bromide. Lanes
containing heavily nicked DNA samples (lanes 7-9, Fig. 3) contained 1.6 μg DNA, to provide sufficient amounts for analysis of remaining covalently closed DNA forms.

**Adaptation of bacteria to alkylation resistance**

The adaptive response to alkylation agents was induced in *E. coli* F26 by exposure to 1 μg·ml⁻¹ N-methyl-N'-nitro-N-nitrosoguanidine (22). After 90 min at 37°, aliquots of the adapted culture and a non-adapted control culture were challenged for 10 min at 37° with several different doses of BCNU or nitrogen mustard, followed by dilution and plating to determine the cytotoxicity of the drugs.

**RESULTS**

**Repair of 6-hydroxyethylguanine residues**

6-Methylguanine-DNA methyltransferase removes methyl groups and (at a slower rate) ethyl groups from the 6 position of guanine in DNA (11,23). In order to investigate if the enzyme is also able to remove substituted ethyl groups from the 6 position of guanine, DNA treated with HNU was employed as a substrate. This alkylating agent was not available in radioactively labeled form. However, 6-alkylguanines are strongly fluorescent, so the fate of the alkylated base could be followed by analysis of DNA hydrolysates with HPLC and fluorescence detection of eluants.

A chromatogram of an acid hydrolysate of the alkylated DNA is shown in Fig. 1a. The sample contained similar amounts of the weakly fluorescent adduct N⁷-hydroxyethylguanine (1.1% of the total guanine residues) and the strongly fluorescent adduct 6-hydroxyethylguanine (0.8% of total guanine). After incubation of the alkylated DNA with the transferase, most of the 6-hydroxyethylguanine had been selectively removed (Fig. 1b). No simultaneous loss of N⁷-hydroxyethylguanine was observed (Fig. 1), as expected from the inability of the transferase to act on N-alkylated residues. The reaction between DNA 6-hydroxyethylguanines and the transferase reached 50% alkyl group removal in 10-15 min at 37° (Fig. 2). We estimate that the enzyme removes hydroxyethyl groups from the 6 position of guanine residues 3 times more slowly than unsubstituted ethyl groups, and 300 times more slowly than methyl groups (ref 12, and unpublished data). The reaction with the hydroxyethylated DNA substrate was essentially completed after 1 hr at 37°; an apparent 1:1 stoichiometric relationship between the amount of hydroxyethyl groups transferred from the
Figure 1. Purine analysis of HNU-treated DNA by HPLC and fluorescence detection. The amount of material in each peak was estimated by comparison with the fluorescence intensities of reference solutions of the various purine derivatives. (a) Left panel: 0.1 M HCl hydrolysate of 40 μg alkylated DNA. The peak of the strongly fluorescent he\(^6\)G only represents 0.8% of the G residues. An \(0^6\)-ethylguanine reference appeared after 60 min, completely separated from he\(^6\)G. (b) Right panel: The same amount of alkylated DNA, incubated with \(0^6\)-methylguanine-DNA methyltransferase (4 μg) at 37° for 1 hr prior to hydrolysis and analysis.

\[
\text{he}^6\text{G} = \text{0}^6\text{-hydroxyethylguanine}; \quad \text{he}^7\text{G} = \text{N}^7\text{-hydroxyethylguanine}.
\]

\(0^6\) position of guanine, and the amount of transferase added, was observed. Thus, with 67, 133 and 200 pmoles transferase in reaction mixtures, 69, 115 and 176 pmoles \(0^6\)-hydroxyethylguanine were removed. These data indicate that the transferase acts in the same way on a substituted ethyl group as on an unsubstituted ethyl or methyl group at the \(0^6\) position of guanine in DNA, with transfer of the alkyl group to a cysteine residue and concomitant suicide inactivation of the enzyme (12,23).

\(0^6\)-Hydroxyethylguanine is generated in DNA not only by HNU and similar hydroxyethylating compounds, but also by agents such as BCNU. In the latter case, the derivative occurs in addition to chloroethylated base residues, due to the decomposition of the nitrosourea by an alternative route involving cyclization instead of chloroethyl cation formation (20,24).
Figure 2. Kinetics of repair of $^{6}\text{O}$-hydroxyethylguanine residues in DNA. The reaction mixture contained hydroxyethylated DNA (240 µg) and $^{6}\text{O}$-methylguanine-DNA methyltransferase (24 µg); 40 µg aliquots of the DNA were removed at various times for analysis as in Fig. 1.

Prevention of DNA interstrand cross-link formation

The substrate specificity of the transferase indicated that it could counteract the appearance of any DNA cross-links that might be expected to occur as a consequence of the formation of reactive intermediates located at the $^{6}\text{O}$ position of guanine. That is, an $^{6}\text{O}$-chloroethylguanine residue in DNA should be converted to guanine, with transfer of the reactive chloroethyl group to the enzyme's acceptor site, before a reaction with the complementary DNA strand could occur. In attempts at an experimental verification, standard methods of measuring interstrand cross-links in purified DNA (6,25) appeared unsuitable, because BCNU generates many monoadducts, apurinic sites, and single-strand breaks in DNA in addition to cross-links. Chain breaks complicate the interpretation of the results when the amount of interstrand cross-links is estimated from the fraction of DNA resistant to strand separation. We have, instead, taken advantage of the observation that after brief exposure to strong alkali (pH > 12.6), covalently closed circular DNA (Form I) collapses into a compact conformation with the strands out of register (Form IV). The Form IV structure cannot be generated from cross-linked circular DNA, because the latter has its complementary strands locked in register and
immediately renatures to the Form I conformation after alkali treatment (26). It was observed here that these two forms of covalently closed circular DNA can be separated from each other, as well as from nicked and single-stranded molecules, by agarose gel electrophoresis. After exposure of closed circular DNA to a cross-linking agent and an alkaline denaturation step, the determined ratio of Form I molecules to Form IV molecules is consequently directly related to the amount of DNA interstrand cross-linking.

In initial control experiments, non-alkylated circular double-stranded M13 DNA was incubated at 37°C for 3 hr with the methyltransferase, or with albumin or reduced glutathione. The albumin served as a control protein without transferase activity, while glutathione was used as an additional control to provide a cysteine-containing peptide. On analysis by gel electrophoresis, the DNA band patterns observed were similar (Fig. 3, lanes 1-3) and indicate that the methyltransferase preparation was essentially free from nuclease or topoisomerase activity. (A minor part of the covalently closed circular DNA had been nicked, due primarily to the presence of dithiothreitol in the reaction buffer). Similar data (not shown) were obtained after addition of BCNU to the reaction mixtures, except that the relative proportion of nicked DNA increased as a function of the BCNU concentration. When the untreated DNA samples were alkali-denatured prior to electrophoresis, the covalently closed DNA collapsed into the faster migrating Form IV (Fig. 3, lanes 4-6; see also reference markers in lanes 10-13). Nicked circular DNA aggregated under the high-ionic strength conditions of the denaturation treatment (26) and remained in the gel wells during electrophoresis. The corresponding BCNU-containing reaction mixtures are shown in Fig. 3, lanes 7-9. In reaction mixtures containing albumin or glutathione (lanes 7 and 8), a clearly detectable fraction of the covalently closed circular DNA remained as cross-linked material at the position of Form I molecules, whereas the non-cross-linked molecules migrated as Form IV. Considerable BCNU-induced chain breakage had occurred in all three samples, as seen from the large proportion of material remaining at the origin. Analysis of radioactively labeled circles showed that with 0.5 mM BCNU (lanes 7 and 8), 30% of the closed circular DNA was cross-linked. (The two-fold higher fluorescence of Form I than of Form IV resulted in visible bands of similar intensity). In separate experiments, the relative proportion of cross-linked, closed circular DNA was observed to increase as a function of the BCNU concentration in the
Figure 3. Agarose gel electrophoresis of circular DNA exposed to BCNU and alkyltransferase. Lanes 1-10 and 13 contain double-stranded M13 DNA. The material in lanes 1, 4 and 7 was incubated for 3 hr at 37° with bovine serum albumin, that in lanes 2, 5 and 8 with reduced glutathione, and that in lanes 3, 6 and 9 with E. coli $O_6$-methylguanine-DNA transferase. The DNA in lanes 1-3 was analyzed in its native form, the DNA in lanes 4-6 after alkaline denaturation, and the DNA in lanes 7-9 after inclusion of 0.5 mM BCNU during the 3 hr incubation period, followed by alkaline denaturation. The reference markers included alkali-denatured double-stranded closed circular DNA, Form IV, (lane 10), single-stranded circular DNA (lane 11), nicked circular DNA (Form II) after alkali treatment (lane 12), and native closed circular DNA, Form I (lane 13). Linear double-stranded M13 DNA, Form III (not shown), migrated at 0.7 times the rate of Form I, while linear single-stranded M13 DNA migrated at 0.97 times the rate of its circular counterpart and was completely separated from Form IV DNA.

reaction mixtures, but at the same time a decreasing proportion of the total DNA could be recovered in covalently closed form due to the BCNU-induced nicking of the DNA substrate (data not shown). When the reaction mixture containing BCNU and alkyl transferase was analyzed, less than 5% of the closed circular DNA was found to be in cross-linked form (lane 9). This result was confirmed in several independent experiments. Thus, the $O_6$-methylguanine-DNA methyltransferase counteracted the formation of BCNU-induced DNA cross-links. The transferase was present in excess in order to remove all the monoadduct intermediates, recognized by the enzyme, formed during the entire 3-hour incubation period. At the end of the reaction, active transferase (5-10% of the initial activity) was shown still to be
Figure 4. Agarose gel electrophoresis of circular DNA exposed to nitrogen mustard and alkyltransferase. Samples were applied as in Fig. 3. Thus, the material treated with nitrogen mustard (0.2 mM) is seen in lanes 7-9, with lane 9 representing the alkyltransferase-containing sample.

present by standard enzyme assays (12). Most of the enzyme inactivation observed could be ascribed to the suicide reaction mechanism of the transferase and to the prolonged incubation at 37° in a reaction mixture that contained an insufficient amount of dithiothreitol to completely stabilize the activity (higher dithiothreitol concentrations caused excessive nicking of the circular DNA in the reaction mixture). Thus, the transferase did not appear to be particularly susceptible to inactivation by the alkylating agent. Direct alkylation of the enzyme by BCNU, moreover, would not have significantly affected the drug concentration in the experiment, since the molar concentration of this reagent was 2000-fold higher than that of the transferase in the reaction mixture.

In separate experiments, methyltransferase (or albumin or glutathione) was added at the end of a 3-hour DNA cross-linking period with BCNU and then incubated for 20 min. No selective removal of cross-links by the transferase could be detected in this case. These data indicate that the enzyme does not act on DNA once the cross-linked bonds are formed, but only on a monomeric reaction intermediate.

Analogous experiments were performed with nitrogen mustard instead of BCNU as the DNA cross-linking agent. In this case, there was no detectable
prevention of cross-link formation by the methyltransferase (Fig. 4). Analysis of the radioactive bands of Forms I and IV of the nitrogen mustard-treated samples revealed 30-35% cross-linking whether in the presence of albumin, glutathione, or transferase. It was again verified by enzyme assays on the nitrogen mustard-containing reaction mixture that active transferase remained in excess at the end of the reaction. Although this experiment does not define unequivocally the location of the cross-link, it is in agreement with the concept that nitrogen mustard-induced interstrand cross-links are mainly initiated at the N7 position of guanine (2,3), since the O6-methylguanine-DNA methyltransferase does not remove alkyl groups from this site in DNA.

**Cytotoxicity in bacteria**

Adaptation of E.coli to resist alkylating agents (22) causes the induction of at least two different DNA repair enzymes (27,28). The ability to repair O6-alkylguanine residues, as well as several other lesions, is greatly improved in such adapted cells. However, little or no active repair of N7-alkylguanines is observed. On challenge of unadapted and adapted E.coli cultures with the cross-linking alkylating agents investigated here, it was found that adapted cells were more resistant to BCNU-induced killing than control cells (24% vs. 10% survival after challenge with 13 mM BCNU). On the other hand, both adapted and unadapted cultures showed the same sensitivity to nitrogen mustard (10% vs. 10% survival after challenge with 1.8 mM nitrogen mustard). These observations are consistent with the experiments on purified DNA, and they lend support to the model whereby lethal DNA cross-links are generated from O6 guanine sites with BCNU, vs. from N7 guanine sites with nitrogen mustard. Several repair functions are induced in the adaptive response to alkylating agents, however, and these data on cells treated with cross-linking agents cannot be considered to be entirely conclusive.

**DISCUSSION**

The present results show that chloroethylnitrosourea-induced interstrand cross-linking of DNA proceeds via an alkylated monomeric reaction intermediate, which can be repaired by purified O6-methylguanine-DNA methyltransferase. This is in general agreement with the model for DNA cross-linking suggested by Kohn (5). The reagent enzyme acts on O6-alkylguanine and O4-alkylthymine in DNA but not on N-alkylated purines or pyrimidines, or on O2-alkylpyrimidines (12; McCarthy et al., in
Figure 5. Three possible structures of DNA interstrand cross-links generated by chloroethylnitrosourea treatment. The -CH$_2$CH$_2$- bridge could be localized between (i) guanine $^6$ and cytosine $^4$, with the guanine residue as the initial alkylation target; (ii) guanine $^1$ and cytosine $^3$; (iii) thymine $^4$ and adenine $^6$, with the thymine residue as the initial alkylation target.

preparation). Three possible alternatives for cross-link formation may be proposed (Fig. 5). After initial alkylation at either the $^6$ position of guanine (structure i) or at the $^4$ position of thymine (iii), the chloroethylated base might react directly with its complementary base to form a -CH$_2$CH$_2$- bridge. No such cross-linked residues have been detected by chemical analysis (10), but it is unclear whether this reflects their absence from BCNU-treated DNA, or technical difficulties in their isolation. The cross-link (ii) shown in Fig. 5 has been demonstrated recently in BCNU-treated DNA by Tong et al. (10). These authors have proposed that it reflects cross-linking following initial alkylation at either the $^1$ position of guanine or the $^3$ of cytosine, or at the $^6$ of guanine followed by an internal cyclization to the $^1$ site. The first two alternatives can now be ruled out (assuming that the cross-link (ii) represents a major interstrand species), since the intermediates would not be susceptible to the transferase. Moreover, the $^3$ site of cytosine and
the N¹ site of guanine are shielded from alkylation in double-stranded DNA (29). On the other hand, the model involving a rearrangement from the guanine 0⁶ position is in good agreement with the present data, and it would also nicely explain the slowness of the cross-linking reaction (5,6), as well as the apparent inability of the 0⁶-methylguanine-DNA methyltransferase to break completed cross-links. Further, it is supported by the observation that guanine-cytosine-rich DNA is cross-linked to a greater extent than adenine-thymine-rich DNA by BCNU treatment (6). It should be emphasized, however, that the occurrence of either, or all, of the three structural alternatives shown in Figure 5 would be compatible with the results presented here.

The previous circumstantial evidence for involvement of a guanine 0⁶ adduct during chloroethylnitrosourea-induced cross-linking of DNA has been based mainly on the inability of mammalian cells of the Mer⁻ (or Mex⁻) phenotype to repair 0⁶-alkylguanine in DNA (30,31), and the associated increased susceptibility of these cells to cross-linking agents (7). However, whereas Mer⁻ cells have been shown to differ from normal cells in their 0⁶-methylguanine-DNA methyltransferase activity (32,33), it is not known if this is the only difference between Mer⁺ and Mer⁻ cells. It has recently been observed that treatment of repair-proficient mammalian cells with N-methyl-N'-nitro-N-nitrosoguanidine causes increased susceptibility to chloroethylnitrosourea-induced cross-linking, possibly by the depletion of the cellular repair enzyme that acts on 0⁶-alkylguanine (8). It is noteworthy that these data implicating the 0⁶ position of guanine in BCNU-induced cross-linking (7,8) are equally compatible with cross-linking through the 0⁴ position of thymine, since the same transferase repair enzyme can act on both sites (McCarthy et al., in preparation). Although 0⁶ guanine adducts are more frequent than 0⁴ thymine adducts (29), both kinds of reactions may occur to a significant extent.

The alkyl transferase employed here was obtained from an E.coli strain that over-produces the enzyme (11). A transferase with apparently identical biochemical properties has been found in mammalian cells, but it is only available in small quantities, and has not yet been purified to a homogeneous form, making it less useful as a reagent (33-35). It may be concluded from the present results that the alkyltransferase function can serve to counteract the mutagenic effect of hydroxyethylating agents, as well as the cell-killing effect of chloroethylnitrosoureas. The unusual
property of the enzyme of undergoing irreversible inactivation on reaction with its substrate, however, implies that it is easily consumed and therefore only effective as a cellular defence at low levels of DNA alkylation.

ACKNOWLEDGEMENT

We thank Dr. W. Lijinsky and Dr. D. Paton for generous gifts of reagents.

*Present address: University Department of Radiotherapy and Clinical Oncology, Newcastle-upon-Tyne, UK

REFERENCES