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INHIBITION OF THE HEPATIC O^6-ALKYLGUANINE–DNA ALKYLTRANSFERASE *IN VIVO* BY PRETREATMENT WITH ANTINEOPLASTIC AGENTS

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Abstract—The mammalian DNA repair enzyme O^6-alkylguanine–DNA alkyltransferase (AT) is inactivated during the repair process and its activity can only be restored by *de novo* synthesis. We have made use of this property to determine whether and to what extent various chemotherapeutic agents alkylate DNA in the O^6-position of guanine, ie. produce lesions susceptible to AT repair. Adult female Fischer rats received a single i.p. injection of a high dose (LD_{50}) of the respective agent and, 5 hr later, a chasing dose of *N*-nitroso-[^{14}C]dimethylamine (0.2 mg/kg; 4 hr survival). The amount of 7-[^{14}C]methylguanine formed was approximately 95 μmol/mol guanine and not significantly altered by pretreatment with any of the drugs. The ratio of O^6-[^{14}C]methylguanine/7-[^{14}C]methylguanine was 0.019 for control animals, indicating that during the observation period of 4 hr, 83% of the O^6-[^{14}C]methylguanine produced had been removed by the hepatic AT. Little or no effect was found in rats that received spirohydantoin mustard, hexamethylmelamine, *cis*-platinum or mitomycin C. A significant increase in the O^6-/7-[^{14}C]methylguanine ratio was found after pretreatment with AZQ (0.026) and cyclophosphamide (0.028), agents for which lesions involving the O^6-position of guanine have not yet been identified. *N*-(2-Hydroxyethyl)-*N*-nitrosourea and the cytostatic haloethylnitrosoureas, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea (PCNU), and *N*-chloroethyl-*N*-hydroxyethylnitrosourea (HECNU) inhibited the hepatic AT, producing a ratio of 0.025–0.035. Considerably higher ratios of 0.059 and 0.101 were observed after administration of the methylating agents procarbazine and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide (DTIC), respectively. Complete saturation of the repair system (O^6-/7-[^{14}C]methylguanine ratio, 0.11) was only achieved with *N*-methyl-*N*-nitrosourea.

Although considerable progress has been made in the identification of DNA adducts resulting from interaction with antitumour drugs, the precise mechanism of action of several chemotherapeutic agents currently in use is still incompletely understood. There is evidence that most of these are metabolized to form reactive intermediates which attack nucleophilic sites in cellular macromolecules and that their cytotoxic and mutagenic effects are due to DNA binding [1]. The biological consequences of base modification include mispairing during DNA replication, single-strand breaks and DNA crosslinking [2, 3]. Of the various adducts formed by alkylating agents, *O*-alkylated DNA bases are considered to be responsible for both cytotoxic and carcinogenic effects. Deficient repair of O^6-alkylguanine often correlates with increased susceptibility to malignant transformation and sensitivity to the cytostatic effects of alkylating drugs [4–6].

Chemical characterization of these DNA adducts has not always been successful. One way of determining whether an unknown compound reacts with DNA at the O^6-position of guanine is based on the observation that the repair of O^6-methylguanine is mediated by a transferase which stoichiometrically transfers the methyl group to one of its own cysteine residues. This process results in inactivation of the enzyme [7, 8]. When the available transferase molecules have been exhausted, *de novo* synthesis is necessary to restore the activity, a process that requires several days *in vivo*. Accordingly, administration of a compound leading to the formation of O^6-alkylguanine in DNA reduces the ability of rat liver to remove labeled O^6-methylguanine formed by a subsequent tracer dose of a methylating agent, e.g. *N*-[^{3}H]methyl-*N*-nitrosourea [9] or [^{14}C]-NDMA§ [10]. The AT is not specific for methyl groups; at a slower rate it can also transfer larger groups such as ethyl [11, 12], hydroxyethyl [13, 14] and chloroethyl moieties, so that a large variety of DNA base adducts can inactivate the enzyme.

In the present study, various antineoplastic agents, including haloethylnitrosoureas, mustards, quinones, hydrazine, triazeno and *cis*-platinum derivatives, were given as a single high dose (LD_{50}) to rats to determine to what extent they produce

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§ Abbreviations used: AZQ, 3,6-diaziridinyl-2,5-bis(carboethoxy-amino)-1,4-benzoquinone; HECNU, 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea; PCNU, 1-(2-chloroethyl)-3-(2,6-dioxo-3-piperidyl)-1-nitrosourea; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; [^{14}C]-NDMA, *N*-nitroso[^{14}C]dimethylamine; hexamethylmelamine, *N,N,N′,N′,N″,N″*-hexamethyl-1,3,5-triazin-2,4,6-triamin; *cis*-platinum, *cis*-diammine-dichloroplatinum (II); DTIC, 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide; AT, O^6-alkylguanine–DNA alkyltransferase.

hepatic DNA lesions that are recognized by the mammalian alkyltransferase.

MATERIALS AND METHODS

Chemicals. [^{14}C]NDMA (54 mCi/mmol) was purchased from NEN Chemicals GmbH (Dreieich, F.R.G.). The radiochemical purity was determined by high pressure liquid chromatography on Lichrosorb RP-18 columns eluted with 10% (v/v) aqueous methanol, and found to be greater than 98%. The chemotherapeutic agents were obtained from Syntex Pharm AG (Allschwil, Switzerland) (mitomycin C); Bristol Myers, S.A.E., (Madrid, Spain) (*cis*-platinum, BCNU); the National Cancer Institute (Bethesda, MD) (AZQ, PCNU, cyclophosphamide, spirohydantoin mustard) and from Miles (Dome) GmbH, Frankfurt (F.R.G.) (DTIC). Procarbazine was supplied by Hoffman-La Roche (Basel, Switzerland). *N*-Nitrosomethylurea was synthesized by Dr. Wiessler, Deutsches Krebsforschungszentrum, (Heidelberg, F.R.G.). HECNU was a gift from Dr. G. Eisenbrand, Fachbereich Lebensmittelchemie und Umwelttoxikologie, Universität Kaiserslautern, Kaiserslautern, F.R.G. Sephabsorb HP was purchased from Pharmacia Fine Chemicals (Sweden). Lumagel SB scintillation cocktails were from Lumac, (Schaesberg, The Netherlands). Ribonuclease A (bovine pancreas, Typ I-A) and cremophor EL were from the Sigma Chemical Company (St. Louis, MO).

Animals. Young adult female Fischer 344 rats (120–150 g body weight; Charles River Wiga, F.R.G.) were maintained on a standard laboratory diet with water *ad libitum*.

Animal experiments. Rats were first treated with a single i.p. injection of the unlabelled antineoplastic agent at a high dosage (LD_{50}). The compounds were dissolved in either water (mitomycin C, 0.5 mg/ml; HECNU, 5 mg/ml, cyclophosphamide, 0.5 mg/ml), 50% DMSO in 0.1 M phosphate buffer, pH 6.5 (AZQ, 1 mg/ml), dimethylacetamide containing polyethylene glycol (PCNU, 9.7 mg/ml), dimethylacetamide containing 10% liposyn II (spirohydantoin mustard, 1.3 mg/ml), 10% cremophor EL (BCNU, 2.5 mg/ml), 0.1 M citrate, 0.2 M phosphate buffer, pH 6.0 (*N*-nitrosomethylurea, 20 mg/ml; *N*-nitroso-(2-hydroxyethyl)urea, 6 mg/ml); aqueous 1% (w/v) citrate, 0.5% (w/v) mannite solution, pH 3.0 (DTIC, 39 mg/ml) or 0.9% saline (procarbazine, 77 mg/ml). Hexamethylmelamine was suspended in water and orally administered. Five hours later, animals received a single i.p. injection of [^{14}C]NDMA (0.2 mg/kg; 54 mCi/mmol). Another 4 hr later they were anesthetized with ether and their livers were rapidly removed, frozen in liquid N_2 and stored at −70°.

DNA extraction. Hepatic DNA was isolated from individual animals using a modification of the phenol method [15]. Briefly, livers (2 g) were homogenized in 10 vol. of sodium dodecyl sulfate buffer (1% sodium dodecyl sulfate, w/v, in 10 mM Tris–EDTA–NaCl, pH 8.0, containing 2 ml of a saturated NaCl solution). Proteins were extracted with 10 vol. of chloroform:*iso*amylalcohol:phenol (24:1:25, v/v/v) by agitation at room temperature for 15 min. The organic and aqueous phases were separated by centrifugation at 2000 *g* for 20 min (4°). The aqueous supernatant was re-extracted with 5 vol. of chloroform:*iso*amylalcohol:phenol (24:1:25, v/v/v). DNA and RNA were precipitated with 2.5 vol. of ethanol:*m*-cresol (9:1, v/v) dried and redissolved in 5 ml water. After addition of 0.25 ml of a saturated sodium acetate solution and 0.6 ml of an RNAse-solution (2 mg/ml, heat-treated for 10 min at 80°), nucleic acids were incubated for 15–20 hr at 4°. The aqueous solution was extracted with chloroform:*iso*amylalcohol (24:1, v/v) and centrifuged at 1500 *g*. Two vol. of ice-cold ethyleneglycol monoethylether were added to the supernatant. The precipitated DNA was washed twice with absolute ethanol and once with ether, and allowed to dry at room temperature. DNA was stored at −70°. Immediately prior to analysis, DNA was depurinated in 0.1 M HCl at 80° for 30 min, neutralized to pH 5–6 and passed through a 0.45 m Gelman ACRO LC13 filter.

Radiochromatography. Purine bases were separated on Sephabsorb HP columns (1 × 50 cm) as previously described [16]. Samples were eluted with 10 mM phosphate buffer (pH 5.5) at a flow rate of 1.4 ml/min (fraction vol., 3.6 ml). Absorbance was determined in individual fractions at 260 nm, using a Shimadzu UV-240 spectrophotometer. Radioactivity was determined after the addition of 6.0 ml of Lumagel SB scintillation cocktail (counting efficiency, 86%). Amounts of alkylated purines were expressed as μmol/mol guanine, assuming that their specific activity was the same as that of one of the methyl groups, i.e. half the specific radioactivity of the injected [^{14}C]NDMA.

RESULTS

Representative Sephabsorb HP chromatograms of hydrolyzed hepatic DNA from rats pretreated with AZQ, procarbazine and DTIC are shown in Fig. 1. The major alkylated purine bases were 7-methyl-

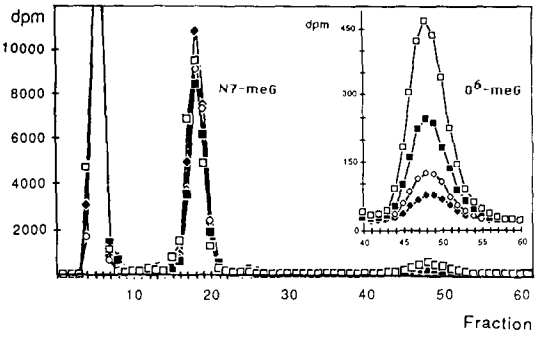

Fig. 1. Chromatography of acid DNA hydrolysates from the liver of rats pretreated with a single i.p. injection (LD_{50}) of various chemotherapeutic agents, followed 5 hr later by an i.p. injection of [^{14}C]NDMA (200 μg/kg; 54 mCi/mmol; 4 hr survival). Superimposed radiochromatographic profiles adjusted to similar amounts of DNA on each column. 7-meG, 7-methylguanine; O^6-meG, O^6-methylguanine. ◆—◆, Control; ◇—◇, AZQ (3 mg/kg); ■—■, procarbazine (200 mg/kg); □—□, DTIC (350 mg/kg).

guanine and O^6-methylguanine. Metabolic incorporation into guanine and adenine was minimal. In rats treated with [^{14}C]NDMA alone, the amount of 7-[^{14}C]methylguanine was 95.7 μmol/mol guanine, with an O^6-[^{14}C]methylguanine/7-[^{14}C]methylguanine ratio of 0.019. The latter showed little variation, with a standard deviation of only 5.3% of the mean. The amount of 7-[^{14}C]methylguanine produced was not significantly altered by pretreatment with any of the drugs although values varied to a greater extent (mean, 90.24 ± 11 μmol 7-meG/mol guanine). In contrast, O^6-[^{14}C]methylguanine values varied significantly, as illustrated in Fig. 1. The amount of O^6-[^{14}C]methylguanine ranged from 1.8 to 10.89 μmol/mol guanine (Table 1). The ratios of O^6-[^{14}C]methylguanine/7-[^{14}C]methylguanine rose to between 0.025 and 0.035 for the haloethylnitrosoureas, AZQ and cyclophosphamide. A considerably higher ratio was obtained with procarbazine (0.059), and DTIC application resulted in a nearly complete saturation (0.101). The highest possible "theoretical" ratio of 0.11 (complete saturation of the AT) was only observed after pretreatment with *N*-nitrosomethylurea. With hexamethylmelamine, spirohydantoin mustard, mitomycin C and *cis*-platinum, the ratio was not significantly different from the control value.

DISCUSSION

Bioactivation of NDMA requires the initial hydroxylation of one methyl group by a microsomal, NADPH-dependent, mixed-function oxidase to form *N*-nitrosomethyl(hydroxymethyl)amine. Formaldehyde is then cleaved off [17] and methanediazonium ion is released as the ultimate methylating intermediate. Rats treated with a single dose of 0.2 mg/kg of [^{14}C]NDMA had hepatic 7-[^{14}C]methylguanine concentrations of 95 μmol/mol guanine. Similar values were obtained after administration of various chemotherapeutic agents (Table 1), indicating that pretreatment did not significantly interfere with the bioactivation of NDMA. Similarly, one can safely assume that due to the short time interval of 5 hr, pretreatment did not change the intralobular distribution of NDMA metabolizing hepatocytes to any significant extent. When methylating *N*-nitroso compounds react with DNA *in vitro*, i.e. in the absence of repair enzymes, the O^6-/7-methylguanine ratio is 0.11 [9]. Since the loss of 7-methylguanine from DNA is negligible during the 4 hr observation period, one can calculate from the very low ratio of 0.019 observed in control animals (Table 1) that during the observation period of 4 hr, 83% of the O^6-[^{14}C]methylguanine produced had been repaired by the hepatic AT. This reflects the remarkably high activity of rat liver AT [18]. The increased ratio observed after pretreatment with various unlabelled chemotherapeutic agents indicates that these compounds form DNA adducts which are recognized by the mammalian AT. Overloading of the hepatic alkyltransferase system [9] in rats can only be expected when the amount of O^6-methylguanine exceeds the value of approximately 180 μmol/mol guanine [19]. In the present study, saturation was only observed in animals pretreated with 95 mg/kg of *N*-nitrosomethylurea. This is in agreement with earlier reports showing that this carcinogen produces approximately 30 μmol O^6-methylguanine/mol guanine per 10 mg/kg [20]. The mammalian AT repairs O^6-methylguanine most efficiently but also acts on O^6-ethyl-, O^6-hydroxyethyl-, O^6-*n*-propyl- and O^6-*n*-butylguanine in DNA, although at markedly reduced rates [13, 14, 21]. *In vivo* experiments have shown that the AT removes O^6-ethylguanine at a 3.5 times slower rate than O^6-methylguanine [21]. Irrespective of the rate of repair, these O^6-alkylguanines (including the free base) all

Table 1. Saturation of the hepatic O^6-methylguanine–DNA alkyltransferase *in vivo* by pretreatment with chemotherapeutic agents

Pretreatment	Dose (mg/kg)	O^6-[^{14}C]meG (μmol/mol G)	7-[^{14}C]meG (μmol/mol G)	Ratio O^6-/7-[^{14}C]meG
Control		1.86	95.09	0.019 ± 0.001
Dimethylacetamid*	10	1.43	84.67	0.017 ± 0.002
Hexamethylmelamine	265	1.45	84.21	0.017 ± 0.001
Spiromustine	9	1.50	83.16	0.018 ± 0.001
Mitomycin C	30	1.86	97.91	0.019 ± 0.001
Cis-platinum	8	2.27	102.57	0.022 ± 0.002
HECNU	25	1.75	70.09	0.025 ± 0.001
AZQ	3	2.31	88.89	0.026 ± 0.001
PCNU	30	2.21	80.13	0.027 ± 0.002
Cyclophosphamide	300	2.63	93.84	0.028 ± 0.001
BCNU	30	2.78	86.04	0.032 ± 0.001
Hydroxyethylnitrosourea	18	3.64	105.12	0.035 ± 0.003
Procarbazine	200	5.84	99.33	0.059 ± 0.005
Dacarbazine (DTIC)	350	10.89	107.42	0.101 ± 0.002
N-Methyl-*N*-nitrosourea	95	8.72	80.00	0.109 ± 0.004

Rats were pretreated with LD_{50} doses of unlabelled chemotherapeutic agents and 5 hr later received an i.p. dose of [^{14}C]NDMA (0.2 mg/kg, 54 mCi/mmol, 4 hr survival). Concentrations of 7-[^{14}C]methylguanine (7-meG) and O^6-[^{14}C]methyl-guanine (O^6-meG) are expressed as fraction of the parent base guanine (G).

* Dimethylacetamide was used as vehicle (see Methods section).

bind to the acceptor site and thus inactivate the enzyme [22]. In contrast to the alkyltransferase isolated from *E. coli*, the mammalian enzyme does not recognize O^4-alkylthymidines [21].

The therapeutic effect of the haloethylnitrosoureas is mainly based on the formation of DNA interstrand cross-links. These agents (e.g. BCNU) yield a chloroethyl cation which attaches to the O^6-position of guanine. The haloethyl group then bridges internally to the N-1 position of guanine, forming an unstable intermediate, 1-O^6-ethanodeoxyguanosine, which subsequently forms an ethane bridge with the N-3 position of deoxycytidine in the opposite DNA strand. The resultant dinucleoside cross-link adduct would thus be 1-(3-deoxycytidyl)-2-(1-deoxyguanosyl)ethane [23]. Since crosslink formation requires 6–12 hr, repair of the O^6-chloroethylguanine lesions by the AT prevents crosslinking and it has been shown that tumor cell lines with a high AT activity are usually less susceptible to the cytotoxic effect of this class of drugs [4–6]. In addition to chloroethylated bases and dideoxynucleoside crosslink adducts, BCNU and related agents also yield a hydroxyethylating species, probably upon decomposition of cyclic intermediates. Since both O^6-chloroethyl- and O^6-hydroxyethylguanine are substrates for the mammalian AT, all haloethylnitrosoureas used in the present study inhibited the repair of O^6-methylguanine produced by a chasing dose of [^{14}C]NDMA, with O^6-/7-[^{14}C]methylguanine ratios in the range of 0.025–0.035. *N*-hydroxyethyl-*N*-nitrosourea and BCNU showed stronger inhibitory effects than HECNU although the latter was found more effective in inducing interstrand crosslinks than BCNU [24, 25]. The rather low O^6-/7-[^{14}C]methylguanine ratio observed (0.025) suggests that the cytotoxicity of HECNU may not be entirely based on interstrand crosslinks that result from the formation O^6-chloroethylguanine.

Mitomycin C and AZQ both function as bioreductive alkylating agents, i.e. an alkylating intermediate is formed after reduction *in vivo* [26, 27]. It has been proposed that mitomycin C is reduced to the respective hydroquinone. Elimination of methanol then yields an indole which can function as alkylating agent (mitosene) or undergo further degradation [28]. Several mitomycin C adducts have been identified in cellular DNA following *in vitro* [29] and *in vivo* [30] exposure. After acid hydrolysis, a base adduct was found with the C1 position of mitosene linked to the O^6-position of guanine. In the present study, no inhibition of the hepatic AT was observed. This could be due to the very low extent of reaction at the O^6-position or an instability of the reaction product. In addition, the possibility exists that the bulky mitosene–DNA adduct may not effectively bind to the acceptor site of the AT. AZQ can undergo a reduction–oxidation cycle of the quinone moiety. This generates free radicals which could produce DNA damage, especially DNA strand scissions. The semiquinone radicals may, however, not be reactive enough to produce covalent bonds with DNA. The interstrand-crosslinks presumably arise by a bifunctional alkylation mechanism involving the two aziridine groups [31–33]. The present study strongly suggests that the O^6 position of guanine is involved in DNA binding by AZQ.

Cis-platinum is a neutral square-planar coordination complex with two labile chloride groups and two relatively inert amine ligands in the *cis*-configuration. In aqueous solutions, it undergoes hydrolysis to form a variety of partially and fully hydrolyzed intermediates [34]. The principal sites of reaction are N7 on purine bases and N3 on pyrimidines. Cross-links occur preferentially between N7–N7 of adjacent guanines [35]. Platinum chelation between the N7 and O^6 atoms of guanine has not been reported. The present study does not provide evidence for the presence of DNA lesions that are recognized by the mammalian AT. However, we cannot determine whether the extent of reaction at the O^6-position of guanine is too small or, as is more likely, the resultant adduct is not susceptible to AT repair.

Bioactivation of cyclophosphamide by the hepatic cytochrome P-450 yields 4-hydroxycyclophosphamide, which in turn decomposes to acrolein and phosphoramide mustard, the ultimate alkylating agent [36]. Following *in vitro* incubation with rat liver microsomes, *N*-(2-chloroethyl)-*N*-[2-(7-guaninyl)-ethyl]amine (NOR-G), *N*-(2-hydroxyethyl)*N*-[2-(7-guaninyl)ethyl]amine (NOR-G-OH), and (to a minor extent) G-NOR-C were identified as DNA adducts [37]. Preferred alkylation site was the N-7 position of guanine. Our studies strongly suggest that the O^6-position of guanine is also involved in the interaction of electrophilic cyclophosphamide intermediates with cellular DNA.

The mechanism of action of hexamethylmelamine, a synthetic s-triazine derivative with cytostatic activity against a wide variety of solid human tumors is uncertain. In rats and humans, hexamethylmelamine undergoes sequential oxidative *N*-demethylation by cytochrome P-450 enzymes. This leads to the formation of hydroxymethylpentamethylmelamine which appears to react with DNA and proteins [38, 39]. In mitochondria, this metabolite is presumably stable whereas in microsomal systems it is rapidly converted to pentamethylmelamine and formaldehyde. $N_2N_4N_6$-Trimethylmelamine, N_2N_4-dimethylmelamine, monomethylmelamine and melamine were identified as urinary metabolites. None of the intermediates showed alkylating activity [40]. Accordingly, the present experiment showed no evidence of a DNA lesion that inhibits the hepatic AT.

Spirohydantoin mustard has been shown to be active against brain tumors in rats and mice [41]. It has little or no genotoxic activity by itself but is metabolized to mutagens by microsomal NADPH-dependent pathways and some DNA cross-linking activity was found [42, 43]. Our experiments revealed no evidence for the formation of DNA lesions involving the O^6-position of guanine.

The relatively high O^6-[^{14}C]methylguanine/7-[^{14}C]methylguanine ratios for procarbazine (0.059) and DTIC (0.101) are not surprising. One of the major metabolic pathways of procarbazine is initiated by oxidation to azo and N_2-azoxy derivatives followed by enzymatic hydroxylation, which yields methyldiazonium hydroxide and benzylic aldehyde

agent [44]. Studies on the extent of DNA methylation in rats have revealed a high methylating capacity [45]. Following a single i.p. dose of procarbazine (110 mg/kg, survival 4 hr), 224 μmol 7-methylguanine/mol guanine and 9 μmol O^6-methylguanine/mol guanine have been determined in rat livers. The amount of O^6-methylguanine formed in the present experiment (200 mg/kg) proved to be sufficient to significantly inhibit the hepatic AT. Bioactivation of dacarbazine (DTIC) requires hydroxylation of one of the methyl groups by a microsomal NADPH-dependent mixed-function oxidase to form 5-(hydroxymethyl-3-methyl-1-triazeno)imidazole-4-carboxamide. After the loss of formaldehyde, the remaining 5-(3-methyl-1-triazeno)imidazole-4-carboxamide spontaneously tautomerizes and eliminates methanediazonium ion as the ultimate carcinogen [46]. Following a single i.p. dose of dacarbazine in rats (9.8 mg/kg, 5 hr survival), 35 μmol 7-methylguanine and 0.7 μmol O^6-methylguanine/mol guanine were determined in liver DNA. Since the extent of DNA alkylation was found to increase linearly with dose [47], it is not surprising that the 350 mg/kg injection administered in the present experiment caused an almost complete inhibition of the hepatic alkyltransferase.

In the interpretation of the results obtained we have assumed that the deficient repair of the O^6-methylguanine produced by the chasing dose of [^{14}C] NDMA was due to the consumption of AT molecules during the repair of O^6-methylguanine produced by the respective pretreatment. However, Brent [48] has recently shown that the loss of AT activity may also result from direct reaction with the enzyme, i.e. protein alkylation. He presented evidence that the various haloethylnitrosoureas may differ in their relative capacity to inactivate the AT directly or indirectly. In the present *in vivo* studies these differential effects cannot be distinguished. It is, however, noteworthy that in both studies simple methylating agents (e.g. *N*-methyl-*N*-nitrosourea) were most effective. We have considered the possibility of a depletion of the hepatic AT by mechanisms other than interaction with O^6-alkylguanines or the transferase. Krokan *et al.* [49] have shown that reactive aldehydes of the 4-hydroxy-α,β-unsaturated type and acrolein inhibit the AT in cultured human bronchial fibroblasts *in vitro*. Several attempts have been made to inhibit this repair system to a therapeutically useful extent *in vivo* but, with the exception of administering large doses of methylating agents, none has been successful.

In conclusion, our experiments confirm that toxic doses of agents which *in vivo* are converted into a methylating intermediate (methyl diazonium hydroxide), e.g. *N*-methyl-*N*-nitrosourea, DTIC and procarbazine, largely abolish the activity of the hepatic AT. A clear, though less extensive inhibition of the AT was obtained with hydroxyethylnitrosourea and the haloethylnitrosoureas BCNU, HECNU, and PCNU. Furthermore, our studies provide convincing evidence that cyclophosphamide and AZQ, agents for which lesions involving the O^6-position of guanine have not yet been identified, form DNA adducts that are recognized by the mammalian AT. *Cis*-platinum produced only a marginal increase in the O^6-/7-methylguanine ratio whereas hexamethylmelamine, spirohydantoin mustard and mitomycin C presented no evidence of interaction at the O^6-position of guanine in nuclear DNA.

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