Interaction of the Carcinogen 3,3-Dimethyl-1-phenyltriazene with Nucleic Acids of Various Rat Tissues and the Effect of a Protein-free Diet

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SUMMARY

The methylation by 3,3-[³⁴C]dimethyl-1-phenyltriazene of nucleic acids in various rat tissues was investigated. Following a single s.c. injection of 3,3-[³⁴C]dimethyl-1-phenyltriazene (50 mg/kg), approximately 40% of the radioactivity was subsequently exhaled as ¹⁴CO₂. Expiration of ¹⁴CO₂, metabolic ¹⁴C labeling of liver proteins, and formation of 7-[³⁴C]methylguanine, the major reaction product with nucleic acid bases, were completed within about 15 hr. Minor alkylation products detectable in DNA were O⁴-methylguanine and 3-methyladenine. In cytoplasmic RNA, 1-methyladenosine, 3-methylcytidine, and O⁴-methylguanosine were present in addition to 7-methylguanosine. Concentrations of 7-methylguanine were highest in nucleic acids of kidney and liver. Among the other organs investigated (brain, lung, spleen, small intestine), 7-methylguanine levels showed little variation but were 4 to 7 times lower than those in liver and kidney. Feeding of a protein-free diet prior to 3,3-[³⁴C]dimethyl-1-phenyltriazene administration reduced the formation of 7-methylguanine in liver and kidney RNA, whereas in the remaining organs the extent of methylation was markedly increased.

The results are discussed with respect to the significance of methylation at specific sites in nucleic acids for the initiation of malignant transformation and the possible role of 3-methyl-1-phenyltriazene as the systemically distributed proximate carcinogen of 3,3-dimethyl-1-phenyltriazene.

INTRODUCTION

Several dialkylaryltriazenes have been shown to be carcinogenic in rats, neural tissues and kidney being predominantly affected (21). Repetitive administration of DMPT¹ to adult rats causes the development of central and peripheral nervous system tumors in more than 75% of experimental animals (2, 21, 25, 26). There is evidence (20, 23) that DMPT requires enzymic hydroxylation of 1 methyl group as the initial step in a sequence of reactions that eventually leads to the generation of a methylating agent as the possible ultimate carcinogen (Chart 1).

Methylation at the N-7 position of guanine in nucleic acids has been shown to occur after administration of [³⁴C]DMPT in vivo (11) but no quantitative data have yet been reported. We have now determined the time course and extent of methylation of nucleic acids in various rat tissues following a single dose of [³⁴C]DMPT. In addition to 7-methylguanine, several minor alkylation products including O⁴-methylguanine have been identified. We have further observed that a protein-free diet differentially alters the extent of methylation of RNA in various organs.

MATERIALS AND METHODS

Chemicals. DMPT was purchased from Th. Schuchardt, Chemische Fabrik, München, West Germany. [³⁴C]DMPT was synthesized (8, 10) by coupling benzenediazonium fluoroborate with equimolar amounts of [³⁴C]dimethylamine hydrochloride (Hoechst AG, Frankfurt, West Germany). The radiochemical purity of the redistilled product, determined by scanning of thin-layer chromatograms (precoated silica gel plates; Merck, Darmstadt, Germany; Rf 0.84 in benzene:acetone, 3:1, v/v), was better than 99%. The yield of [³⁴C]DMPT was 70 to 73% of the theoretical; the specific radioactivity was 241 and 296 μCi/mmol (10).

Animal Experiments. Female BD IX rats (1), 120 to 160 g, were maintained on a standard laboratory diet (Aftromin). In some experiments, a protein-free diet was given for 7 days prior to the administration of [³⁴C]DMPT. The bulk of this diet (17) consisted of carbohydrate (65% corn starch, 30% sucrose, and 5% olive oil). The labeled DMPT was injected s.c. as a solution in edible plant oil (Livio), at a maximal injection volume of 0.5 ml.

Exhalation of ¹⁴CO₂. Animals were placed in a metabolic cage that was flushed with air at a flow rate of 2 liters/min. Exhaled ¹⁴CO₂ was determined as BaO⁴CO₃ after absorption in 4 M NaOH (3).

Isolation of Nucleic Acids. Animals were killed by exsanguination under ether anesthesia. Organs were frozen in liquid N₂ and stored at −70°. DNA was isolated by phenol extraction as described earlier (16). Cytoplasmic RNA was prepared from the postmitochondrial supernatant (18).

Hydrolysis of Nucleic Acids and Chromatographic Methods. 7-Methylguanine and minor methylated purine bases in DNA were determined (14, 16) by Sephadex G-10 chroma-
For the determination of 7-methylguanine alone, DNA or RNA samples were hydrolyzed in 1 M HCl (100°, 1 hr), and the hydrolysate was analyzed on Dowex 50-X12 (200 to 400 mesh, H+ form) columns (11 x 0.6 cm) using an exponential 1 to 3 M HCl gradient (5). This chromatographic system was also used for the determination of 7-methylguanine in total nucleic acids following the hydrolysis of tissue homogenates in 5% (w/v) trichloroacetic acid (90°, 15 min) and 1 M HCl (100°, 1 hr) as described earlier (5). Cytoplasmic RNA was enzymically hydrolyzed (7) using pancreatic RNase (EC 2.7.7.16), phosphodiesterase (Crotalus terrificus terrificus, EC 3.1.4.1), and alkaline phosphatase (Escherichia coli, EC 3.1.3.1). Ribonucleosides were then separated on Dowex 50-X4 (minus 400 mesh, NH4+ form) columns (85 x 1 cm) using 0.3 and 1.0 M ammonium formate (pH 8.9) as eluent (7, 14).

Amounts of methylation products were expressed as mole-% of the parent base, using published molar extinction coefficients and assuming that the specific radioactivity was the same as that of 1 methyl group of the injected [14C]DMPT (5, 27).

**Determination of Radioactivity.** Chromatographic fractions were dried in a stream of air and counted for radioactivity after the addition of 0.3 ml Hyamine hydroxide and 10 ml toluene containing 0.5% (w/v) PPO and 0.05% (w/v) bis-MSB. Quench corrections were made by automatic external standardization. Counting efficiency was in the range of 86 to 89%.

**RESULTS**

Following a single dose of 50 mg of [14C]DMPT per kg, about 40% of the injected radioactivity was exhaled as 14CO2. The time course of 14CO2 expiration indicates that the formation of 14CO2 was completed within about 15 hr. The 14C-labeling of liver proteins and the formation of 7-[14C]methylguanine in liver nucleic acids followed a similar time course, with maximum values being reached about 15 hr after the administration of the carcinogen (Chart 2).

At 8 hr after [14C]DMPT injection, 7-methylguanine concentrations in cytoplasmic RNA were highest in kidney and liver (Chart 3). In both organs, 7-methylguanine increased linearly with doses up to 160 mg/kg. At the highest dose level (320 mg/kg), which is approximately the acute LD50 (2), the dose-response curves tended to reach a plateau. In RNA from brain and small intestine, 7-methylguanine concentrations were very similar and markedly lower than those in RNA from kidney and liver. Furthermore, the production of 7-methylguanine did not, as in kidney and liver, level off at the 320-mg/kg dose.

Table 1 gives the 7-methylguanine contents of DNA and RNA for a wider range of rat tissues. The amounts of 7-methylguanine produced by a single dose of [14C]DMPT (100 mg/kg, 8-hr survival time) were approximately 4 to 5 times higher in DNA of kidney and liver than the amounts in spleen, lung, intestine, and brain. In cytoplasmic RNA these differences were even more marked, the 7-methylguanine values being 5 to 7 times higher in kidney and liver than the values in the remaining organs. Feeding of a protein-free diet for 1 week prior to [14C]DMPT administration decreased the production of 7-methylguanine in cytoplasmic RNA of liver and kidney, whereas in all other organs investigated the extent of N-7 alkylation of guanine increased by 50 to 100%.

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When liver DNA from animals treated with [\(^{14}\text{C}\)]DMPT (150 mg/kg, 8-hr survival time) was subjected to mild acid hydrolysis, separation of the purine bases revealed the presence of 3-methyladenine and O\(^6\)-methylguanine as minor alkylation products (Chart 4). In the same experiment, cytoplasmic RNA was enzymically degraded to yield the pyrimidine and purine nucleosides which were then chromatographed on Dowex 50-X4 columns (NH\(_4^+\) form). Under the alkaline conditions of the enzymic hydrolysis, most of the 7-methylguanosine formed is converted into various ring-opened products which eluted between Fractions 5 and 22 (Chart 5). Minor alkylation products detectable were O\(^6\)-methylguanosine and 3-methylcytidine. During enzymic hydrolysis, 1-methyladenosine is partially rearranged to form N\(^\text{\textsubscript{6}}\)-methyladenosine (13) which itself is not known to be a direct methylation product.

**DISCUSSION**

It has been suggested that the initial step in the formation of an alkylating intermediate from DMPT and related dialkylartriazenes is the enzymic hydroxylation of one of the methyl groups which leads to the production of formaldehyde and MPT. The latter has been postulated to be the proximate carcinogen of DMPT (20, 21, 23) which, after hydrolytic fission, yields aniline and methyl diazonium hydroxide (Chart 1). This highly unstable product (or the released methyl carbonium ion) is thought to methylate nucleophilic groups in cellular macromolecules. Supporting evidence for this route of metabolism has emerged from the work of...
Preussmann et al. (23) who, after in vitro incubation of DMPT and other dialkyltriazenes with microsomal fractions from rat liver, demonstrated the production of both formaldehyde and aniline. Spontaneous hydrolysis of DMPT, on the other hand, leads to the formation of benzenediazonium ion and dimethylamine (9). The hypothesis that MPT is the proximate carcinogen of DMPT is based on the carcinogenicity of MPT (20, 24) and its ability to methylate purine bases in DNA (3) and RNA (5) in the absence of drug-metabolizing enzymes (22). In the intact animal, formaldehyde would be expected to enter rapidly the cellular C2 pool, with the 14C atom of [14C]DMPT being incorporated into various intermediary products including purine bases, amino acids, and CO2. The present experiments have indeed shown that as much as 40% of the radioactivity injected as [14C]DMPT is subsequently exhaled as 14CO2 (Chart 2).

The extent of 7-methylguanine formation was highest in nucleic acids of kidney and liver, irrespective of the dose of DMPT applied (Chart 3). In all other organs investigated (Table 1), the concentrations of 7-methylguanine in DNA and cytoplasmic RNA showed little variation, the average value being 4 to 7 times lower than that in liver and kidney. This finding is compatible with the hypothesis that these organs, including the nervous system, are themselves not able to metabolize DMPT to a significant extent and that the methylation of their biopolymers is caused by a proximate carcinogen, possibly MPT. At present, however, only circumstantial evidence is available for the intermediary formation of MPT in rat liver and the results of the present experiment in which 1 group of animals was given a protein-free diet are difficult to reconcile with the hypothesis that MPT is the systemically effective proximate carcinogen of DMPT. Protein-free diet has previously been shown to decrease markedly the activity of drug-metabolizing enzymes and, consequently, the metabolism of carcinogens such as dimethylnitrosamine in rat liver (28). Similarly, this diet reduced the methylation of RNA in liver and kidney by DMPT (Table 1). If MPT were the proximate carcinogen formed in liver and kidney, the lower rate of O-C hydroxylation in animals fed a protein-free diet would be expected also to reduce RNA alkylation in those organs that are not themselves able to demethylate DMPT enzymically. However, the experimental results clearly showed (Table 1) that the inhibition of DMPT metabolism in the liver (and kidney) is paralleled by a significant increase in the alkylation of RNA in lung, intestine, spleen, and brain. A similar relationship was found when the extent of nucleic acid alkylation was investigated as a function of the dose of DMPT administered (Chart 3). In liver and kidney, a linear dose response was observed only at doses up to 160 mg/kg, indicating that saturation of the microsomal dealkyllating system occurred at higher doses (320 mg/kg). In contrast, methylation of nucleic acids in brain and intestine increased approximately linearly over the entire dose range. These results suggest that the formation of the systemically distributed proximate carcinogen does not, as would be expected for MPT, depend on the enzymic hydroxylation of one of the methyl groups of the parent carcinogen. The identity of the DMPT intermediate responsible for the methylation of nucleic acids in organs that themselves are unable to metabolize DMPT remains unknown, but we suggest a stabilized conjugate as a potential transport form. The possibility of degradative pathways additional or alternative to that shown in Chart 1 is also supported by recent mutagenesis studies (15, 19, 29) which showed that DMPT may act as either a direct or an indirect mutagen.

No consistent correlation seems to exist between the extent of alkylation of nucleic acids in different rat tissues and the location of tumors produced by DMPT. The principal target organ in the carcinogenicity of DMPT is the nervous system, but in the brain the extent of methylation of nucleic acids was about 4 times lower than that in the liver, although DMPT has, to our knowledge, never produced liver tumors. However, the kidneys that showed the highest concentrations of 7-methylguanine in nucleic acids are among the organs in which DMPT preferentially induces tumors (21).

It is now generally agreed that N-7 alkylation of guanine in DNA, although a valuable indicator of the overall extent of alkylation, does not play a significant role in the malignant transformation by monofunctional alkylating agents. Minor alkylation products, in particular O-methylguanine, may be even more important. The relative extent of O-alkylation of guanine in DNA produced by different agents closely parallels their carcinogenic potency (12). Furthermore, the location of tumors has been attributed to the differential capacity of various organs for enzylic excision of O-alkylguanine. For example, the neurocogenic effect of N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea has been explained by the fact that the brain, in contrast to the liver and other organs, is not capable of a significant repair excision of O-alkylguanine.

Chart 5. Fractionation of ribonucleosides of cytoplasmic RNA from livers of rats treated with [14C]DMPT (experimental details as in Chart 4). RNA was isolated, enzymically hydrolyzed, and separated on Dowex 50-X4 columns (NH4+ form), using 0.3 M (Fractions 1 to 90) and 1.0 M (Fractions 91 to 130) ammonium formate (pH 8.9; fraction volume, 4.5 ml). AR, uridine; CR, cytidine; GR, guanosine; AR, adenosine. 3′-Methylyadenosine (3′-MeAR), 3′-methylcytidine (3′-MeCR), and 1-methyladenosine (1-MeAR) were added as markers. Alkaline breakdown products of 7-methylguanosine (7-MeGR) elute between Fractions 5 and 22. ●, E1/2; ○, dpm.
alkylguanine from its DNA (4, 6, 16). In the present experiment, O'-methylguanine was found to be produced in rat liver DNA (Chart 4) and RNA (Chart 5), but the low specific radioactivity of the [14C]DMPT used did not allow its quantitative detection in various tissues. In spite of the low extent of alkylation of brain DNA produced by a single dose of DMPT, repetitive administration (which is necessary for the selective induction of nervous system tumors) may lead to a preferential accumulation of O'-methylguanine in cerebral DNA; this has already been demonstrated (16) for the neurooncogenic compound N-methyl-N-nitrosourea.

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