Biochemical Pharmacology, Vol. 20, pp. 529–533. Pergamon Press, 1971. Printed in Great Britain

MECHANISM OF CARCINOGENESIS WITH 1-ARYL-3,3-DIALKYL-TRIAZENES—III

IN VIVO METHYLATION OF RNA AND DNA WITH 1-PHENYL-3,3-[^{14}C]-DIMETHYLTRIAZENE

F. W. KRÜGER, R. PREUSSMANN and N. NIEPELT

Institut für Experimentelle Toxikologie und Chemotherapie am Deutschen Krebsforschungszentrum, Heidelberg and Forschergruppe Präventivmedizin, Freiburg/Br., Germany

(*Received* 14 *April* 1970; *accepted* 23 *July* 1970)

Abstract—Intraperitoneal injection of 1-phenyl-3,3-[^{14}C]-dimethyltriazene to rats resulted in the formation of 7-methylguanine which was isolated from RNA and DNA of the liver; labelled 7-methylguanine was also present in the RNA of brain and kidney. The results show that the carcinogenic 1-phenyl-3,3-dimethyltriazene is metabolized *in vivo* to form an alkylating agent.

RECENT investigations revealed that the carcinogenic 1-phenyl-3,3-dimethyltriazene[1] is metabolized *in vitro* by the microsomal fractions from liver, kidney, and lung in the presence of a NADPH-generating system and oxygen. Since the metabolites detected were formaldehyde and aniline, it was suggested that 1-phenyl-3,3-dimethyltriazene is oxidatively demethylated and the resulting 1-phenyl-3-monomethyltriazene is subsequently cleaved to aniline and methyldiazohydroxide, which is the ultimate donor of a methyl cation.[2] This hypothesis is supported by the fact that phenylmonomethyltriazene was found to alkylate nucleic acids and nucleotides *in vitro* to form 7-methylguanine.[3] Further evidence comes from biological experiments which showed that 1-phenyl-3,3-dimethyltriazene acts as a systemic carcinogen, suggesting enzymatic activation, whereas 1-phenyl-3-monomethyltriazene mainly produces local tumors at the injection site,[4, 5] indicative of a "direct" action.

Experiments were designed to examine whether similar methylation occurs *in vivo*. Therefore [^{14}C]-methyl labelled 1-phenyl-3,3-dimethyltriazene was injected into rats and the isolated nucleic acid bases were examined for the presence of methylated products.

MATERIAL AND METHODS

Synthesis of labelled compound. [^{14}C]-Dimethylamine hydrochloride (20·6 mc/mM) was purchased from the Radio-Chemical Centre, Amersham. 1-phenyl-3,3-[^{14}C]-dimethyltriazene was synthetised by the procedure of Rondestvedt and Davis,[6] which was modified as follows: Freshly distilled aniline (0·250 g; 2·68 m-moles) was dissolved in aqueous HCl (1·25 ml conc. HCl diluted to 5 ml). The ice-cooled solution was diazotized by dropwise addition of sodium nitrite (0·25 g; 3·63 m-moles), dissolved in 0·75 ml of H_2O. After stirring for 0·5 hr, the cooled solution was made alkaline by dropwise addition of sodium carbonate solution (30%) to neutralize excess nitrous

acid and to prevent formation of dimethylnitrosamine in the following steps. [^{14}C]-Dimethylamine hydrochloride (1·96 mg, 500 μc), diluted with 0·215 g of inactive compound was dissolved in sodium carbonate (30%, 2 ml) and the solution was slowly added to the cooled reaction-mixture over 30 min with continuous stirring. Ammonia solution (25%, 20 ml) was added and the resulting 1-phenyl-3,3-[^{14}C]-dimethyltriazene was isolated by steam distillation. The distillate (150 ml) was collected and extracted with ether (5 × 100 ml). The combined extracts were dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to a constant weight of the residue. The yield of 1-phenyl-3,3-[^{14}C]-dimethyltriazene was 350 mg (2·35 m-moles) (88 per cent of the theory). The obtained product showed the typical u.v. absorption maxima at 225, 284, 292 and 305 nm.

Animal experiments. Male Sprague–Dawley rats, maintained on Altromin^{R}-diet and water *ad lib.*, were used. The solution of the carcinogen was prepared by mixing 520 mg/524 μc 1-phenyl-3,3-[^{14}C]-dimethyltriazene with 4 ml Tween 80^{R} (Oleylsorbitole polyethyleneglycol ether, obtained from SERVA, Heidelberg). Thirty-four rats with an average weight of 110–120 g were injected intraperitoneally with 1-phenyl-3,3-[^{14}C]-dimethyltriazene (13·0 mg/136 μc/kg). Therefore each animal was injected with 0·1 ml/100 g body wt. The rats were killed 9 hr and 30 min after treatment.

Determination of 7-methylguanine. RNA and DNA were prepared from pooled organs by the modified method of Kidson, Kirby and Ralph[7] and then hydrolysed at 100° with 1 N HCl for 1 hr. Because of insufficient quantity of the other tissues, DNA was prepared from liver only. The hydrolysate was chromatographed on a Dowex-column 50 WX 2 (H-form)/200–400 mesh and eluted with an HCl gradient, ranging from 1 to 2 HCl. The relative optical density of the fractions was measured with a Beckmann DK 2 A spectrophotometer. After removal of HCl over KOH *in vacuo*, aliquots of each fraction were dissolved in water and the radioactivity was determined with a Beckmann-LS150 liquid scintillation counter, using a dioxan scintillator (Beckmann-Fluorally R). Narrow carbon window with an efficiency of 73 per cent was used and the results were quench-corrected by the external standard.

RESULTS AND DISCUSSION

The results are summarized in Table 1 and Figs. 1–4. The nucleic acids from all tissues were labelled (Table 1). 7-methylguanine was formed in the RNA of all tissues investigated (Figs. 1, 3, 4) and was also found to be present in the DNA of liver (Fig. 2).

TABLE 1. SPECIFIC ACTIVITIES OF RNA AND DNA, ISOLATED FROM ORGANS OF RATS TREATED WITH 1-PHENYL-3,3-[^{14}C]-DIMETHYLTRIAZENE

Dosage (mg/136 μc/kg)	Number of animals	Organs examined	Nucleic acids examined	Specific activity (DPM/mg)	7-methyl-guanine found
130	34	liver	RNA	493	+
			DNA	368	+
130	34	kidney	RNA	555	+
130	34	brain	RNA	194	+

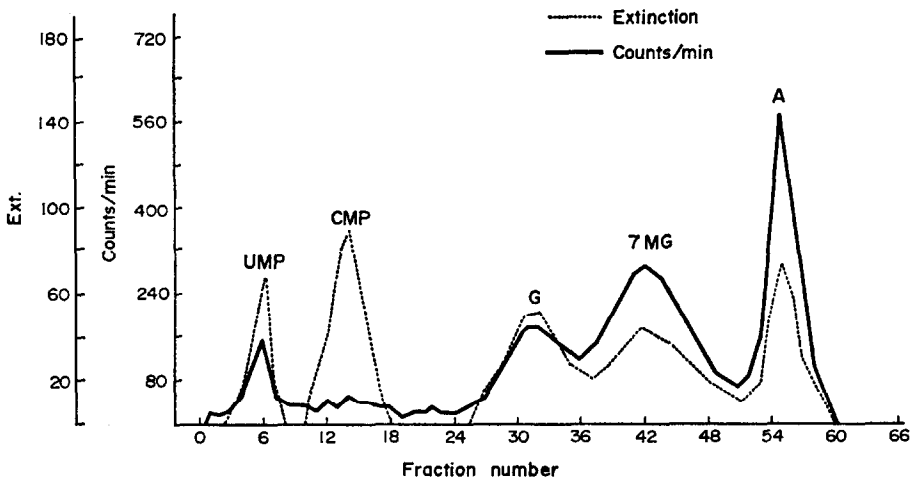

FIG. 1. Chromatographic profiles of acid hydrolysates of liver RNA from rats treated with 1-phenyl-3,3-[^{14}C]-dimethyltriazene. 50 mg RNA, 5 mg carrier 7-MG added. Separation on Dowex 50. UMP = uridine monophosphate, CMP = cytidine monophosphate, G = guanine, 7-MG = 7-methyl-guanine, A = adenine.

The results of these *in vivo* experiments confirm our former results *in vitro*[3, 4] and clearly show that the carcinogenic 1-phenyl-3,3-dimethyltriazene is metabolized in mammals to form an alkylating agent. The same is true for other potent chemical carcinogens, for example the *N*-nitroso compounds[8] and aliphatic hydrazo-, azo- and azoxy compounds.[4]

1-Phenyl-3,3-dimethyltriazene in rats predominantly produces malignant tumors of the brain, the nervous system and the kidney, but according to our experience no tumors in the liver.[1, 5] Therefore, as far as RNA is concerned, there seems to be no direct correlation between organotropic carcinogenic action and the overall methylation of guanine in position 7. Similar observations have been made for some carcinogenic *N*-nitroso compounds.[9–12] However, only alkylation of specific sites might be

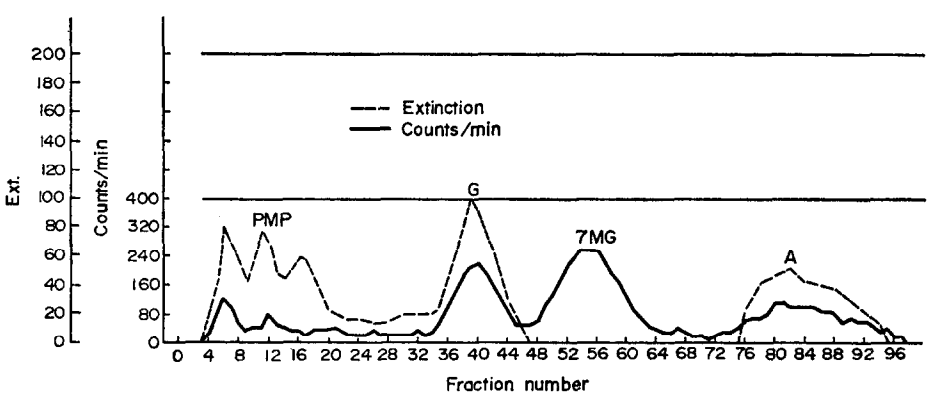

FIG. 2. Chromatographic profiles of acid hydrolysates of liver DNA from rats treated with 1-phenyl-3,3-[^{14}C]-dimethyltriazene. 50 mg DNA. Explanations see Fig. 1, PMP = Pyrimidinmono-phosphates.

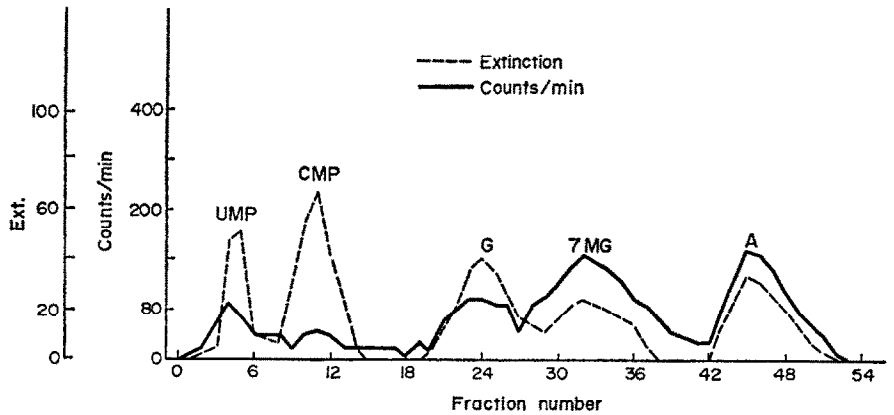

FIG. 3. Chromatographic profiles of acid hydrolysates of kidney RNA from rats treated with 1-phenyl-3,3-[^{14}C]-dimethyltriazene. 18 mg RNA, 1·8 mg carrier 7 MG added. Explanations see Fig. 1.

responsible for the conversion of a normal to a neoplastic cell and this cannot be investigated with methods available at present. On the other hand, no attempt was made in the present qualitative investigation to look for minor alkylation products of purine and pyrimidine bases such as 1- and 3-methyladenine or 6-*O*-methylguanine[13,14] which might also be related with carcinogenesis.

The results presented here are in good accordance with the mechanism of metabolism presented earlier.[2] The relatively high incorporation of activity into purine and pyrimidine bases can be easily explained as resulting from the enzymatically formed formaldehyde via the C-1-pool. After dealkylation 1-phenyl-3-monomethyltriazene should be formed, which is a known alkylating agent[15] and which alkylates nucleic acids and nucleotides *in vitro* to form 7-methylguanine.[3]

Since dimethylnitrosamine is a potent methylating agent *in vivo*,[17] an alternative explanation for the formation of 7-methylguanine might be sought in the presence of small amounts of dimethylnitrosamine in the triazene. Excess nitrite is invariably used

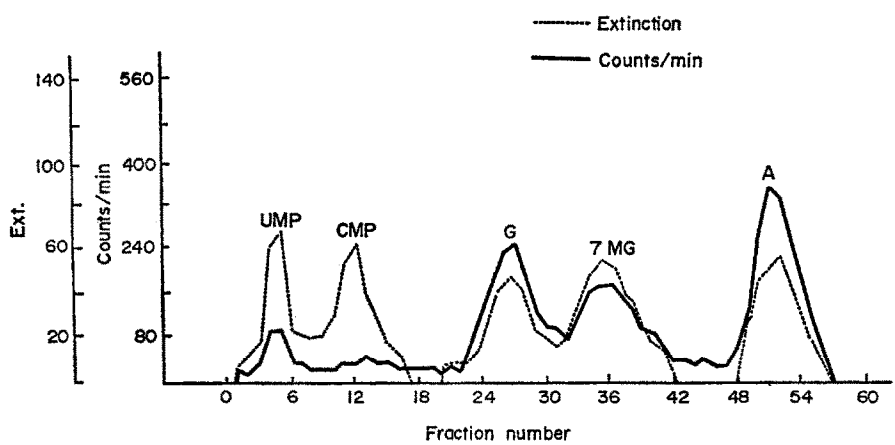

FIG. 4. Chromatographic profiles of acid hydrolysates of brain RNA from rats treated with 1-phenyl-3,3-[^{14}C]-dimethyltriazene. 50 mg RNA, 5 mg carrier 7 MG added. Explanations see Fig. 1.

in the diazotation of aniline, and therefore some nitrous acid could possibly effect nitrosation of dimethylamine which is used as the coupling component in the triazene synthesis. The formation of dimethylnitrosamine is highly improbable under alkaline conditions required for triazene synthesis and since no detectable quantity of dimethylnitrosamine was found in a large-scale preparation of 1-phenyl-3,3-dimethyltriazene (1 mole), synthesized under identical conditions as described in the experimental part for the labelled compound. Nevertheless, a rigorous examination of the labelled triazene for trace amounts of contamination was performed: Direct gas-chromatographic investigation of 1-phenyl-3,3-[^{14}C]-dimethyltriazene, used in the labelling work, showed no peak corresponding to dimethylnitrosamine. In a more rigorous examination 95 mg of the labelled compound was chromatographed by TLC and the zone corresponding to the R_f-value of dimethylnitrosamine was extracted and the residue examined for the presence of dimethylnitrosamine by means of gas chromatography and polarography. Neither method revealed the presence of nitrosamine. These results show that contamination by dimethylnitrosamine, if any, was less than 0·05 μg.

Acknowledgements—Our work has been supported by the Deutsche Forschungsgemeinschaft and the Verein zur Förderung der Krebsforschung in Deutschland e.V. We thank Mr. H. Braun for skilled technical assistance, and Dr. G. Kolar for his help in the preparation of the English manuscript.

REFERENCES

1. H. Druckrey, S. Ivankovic and R. Preussmann, *Naturwissenshaften* **53**, 557 (1967).
2. R. Preussmann, A. v. Hodenberg and H. Hengy, *Biochem. Pharmac.* **18**, 1 (1969).
3. R. Preussmann and A. v. Hodenberg, *Biochem. Pharmac.* **19**, 1505 (1970).
4. R. Preussmann, H. Druckrey, S. Ivankovic and A. v. Hodenberg, *Ann. N.Y. Acad. Sci.* **163**, 697 (1969).
5. R. Preussmann, H. Druckrey, S. Ivankovic, Ch. Landschütz, E. Flohr and U. Griesbach, *Z. Krebsforsch.*, in preparation.
6. C. S. Rondestvedt and S. J. Davis, *J. org. Chem.* **22**, 200 (1957).
7. C. Kidson, K. S. Kirby and R. K. Ralph, *J. molec. Biol.* **7**, 312 (1963).
8. H. Druckrey, R. Preussmann, S. Ivankovic and D. Schmähl, *Z. Krebsforsch.* **69**, 103 (1967).
9. F. W. Krüger, H. Ballweg and W. Maier-Borst, *Experientia* **24**, 592 (1968).
10. W. Lijinsky and A. E. Ross, *J. natn. Cancer Inst.* **12**, 1059 (1969).
11. R. Schoenthal, *Biochem. J.* **114**, 55 (1969).
12. F. W. Krüger, G. Walker and M. Wiessler, *Experientia* **26**, 520 (1960).
13. A. Loveless, *Nature, Lond.* **223**, 206 (1969).
14. P. D. Lawley and C. J. Thatcher, *Biochem. J.* **116**, 693 (1970).
15. O. Dimroth, *Ber. dtsch. chem. Ges.* **36**, 909 (1903); **38**, 670 (1905).
16. P. N. Magee and J. M. Barnes, *Adv. Cancer Res.* **10**, 163 (1967).