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METHYLATION OF LIVER DNA OF RAT AND MOUSE BY *N*-NITROSODIMETHYLAMINE FORMED *IN VIVO* FROM DIMETHYLAMINE AND NITRITE*

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Abstract—The extent of formation of *N*-nitrosodimethylamine (NDMA) in the stomachs of rats and mice after simultaneous oral administration of [^{14}C]dimethylamine and potassium nitrite was determined by measuring the methylation of liver DNA. With doses of around 1 mg dimethylamine hydrochloride/kg body weight and 50 mg potassium nitrite/kg body weight, 0.8% of the amine was nitrosated on average. The individual fluctuations ranged from 0.2 to 1.3% in the rat and from 0.2 to 1.9% in the mouse. Simultaneous administration of 50 mg sodium ascorbate (vitamin C)/kg body weight inhibited the nitrosation by about 80%, while 50 mg α-tocopherol acetate (vitamin E)/kg body weight reduced the nitrosation by about a half. Assuming similar kinetics and conditions of nitrosation in rats and man, a comparison of the formation of NDMA *in vivo* from dietary dimethylamine and nitrite with the estimated human uptake of preformed NDMA revealed that *in vivo* formation in the stomach of man is probably negligible.

INTRODUCTION

The generation of nitrosamines from the reaction of amines with nitrite and their carcinogenicity in animals and man is widely discussed (IARC, 1980). The nitrosation of dimethylamine (DMA) is particularly important because this amine is a ubiquitous dietary constituent and its nitrosation product, *N*-nitrosodimethylamine (NDMA), is a strong carcinogen. The reaction kinetics and pH-dependence, as well as the inhibitors and catalysts, of this nitrosation reaction *in vitro* are relatively well known (Mirvish, 1975), but few quantitative data are available on the extent of NDMA formation *in vivo*. However, such information would be needed for a risk assessment in man. Rounbehler, Ross, Fine *et al.* (1977) determined the NDMA content of whole frozen mice after treatment with DMA and nitrite. Histological damage to the liver from such treatment has also been reported (Asahina, Friedman, Arnold *et al.* 1971; Cardesa, Mirvish, Haven & Shubik, 1974; Pollard, Sharon & Chang, 1972) but in no case was the extent of the nitrosation estimated. Braun attempted to determine the *in vivo* nitrosation of amines using the mutagenic properties of the nitrosamines. However, no mutagenicity was detected in their host-mediated Ames test in mice treated with DMA and equimolar nitrite (Braun, Schöneich & Ziebarth, 1977).

The carcinogenicity of the nitrosamines seems to result from the covalent interaction of their chemically reactive metabolites or breakdown products with DNA in the target cell. NDMA leads to the methylation of DNA at more than a dozen different positions. About 80% of these methylations occur at the nitrogen-7 atom of guanine (Lawley, 1976; Singer, 1975). Determination of this DNA damage could therefore be used as an indicator of nitrosamine formation *in vivo*.

Here, we report the results of a study on the formation of 7-methylguanine in liver DNA of rats and mice after simultaneous oral administration of [^{14}C]dimethylamine and nitrite. The level of DNA methylation was compared with that reached after administration of the presumed nitrosation reaction product, NDMA. We also tested whether this reaction could be inhibited by administration of the antioxidants vitamin C (sodium ascorbate) or vitamin E (α-tocopherol acetate). These substances are often discussed as inhibitors of nitrosamine formation (IARC, 1980). The mechanism of the inactivation of nitrite by vitamin C was studied by Dahn, Loewe & Bunton (1960).

EXPERIMENTAL

Materials. Potassium nitrite, sodium ascorbate and α-tocopherol acetate were purchased from Fluka AG (Buchs). Radioactive compounds were purchased from The Radiochemical Centre, Amersham, Bucks, England. [^{14}C]Dimethylamine.HCl had a specific activity of 54 mCi/mmol and a radiochemical purity of

Abbreviations: DMA = dimethylamine; HPLC = high-performance liquid chromatography; NDMA = *N*-nitrosodimethylamine; TLC = thin-layer chromatography.

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98% as determined by thin-layer chromatography (TLC) on Cellulose F_{254} aluminium foils (E. Merck GmbH, Darmstadt, FRG), using a solvent mixture of isopropanol–ethanol 1 N-HCl (75:75:50, by vol.) as the eluant and ninhydrin reagent for detection. ^{14}C-labelled NDMA had a specific activity of 7.5 mCi/mmol and a radiochemical purity of 96% as determined by TLC on Silica Gel GF_{254} (E. Merck GmbH), using hexane–ether–methylene chloride (40:30:20, by vol.) as the eluant. Hydroxyapatite came from Bio-Rad Laboratories, Richmond, CA (Bio-Gel HTP DNA-grade, selected batches for high yields). Insta-Gel scintillation cocktail was from Packard Instruments Co., Frankfurt, FRG. ^{14}C radioactivity was measured in a Berthold Betaszint 5000 liquid scintillation counter. DNA samples and purine-base fractions were counted in low-background Beckman glass vials. Counting efficiency was determined by the external standard method after calibration with [^{14}C]hexadecane standard.

Animals and treatments. Young adult male rats (ZUR:SIV-Z, Sprague–Dawley derived, 200–300 g) and mice (NMRI, 22–35 g) were used throughout. The animals were housed in macrolone cages and were given water and food (Laboratory chow, pellets No. 890; Nafag AG, Gossau) *ad lib.* Administrations of test solutions by oral gavage (1–1.5 ml and 0.5–1.0 ml for rats and mice, respectively) were always performed between 9 and 10 a.m. After 6 hr the animals were killed by open heart puncture under ether anaesthesia and their livers were removed and processed immediately by homogenization in a Waring blender.

Stock solutions of 50 mg/ml potassium nitrite or sodium ascorbate were prepared in deionized water. α-Tocopherol acetate (500 mg) was dissolved in 3 ml ethanol and mixed with water up to 10 ml to produce a fine suspension. Stock solutions were mixed in small tubes on a balance on the basis of each animal's weight. An aliquot of the radiolabelled compound dissolved in water was added and the exact amount of radioactivity administered was determined by weighing the syringe before and after gavage and by measuring the specific activity of the solution administered. The dose (mg/kg) of the radiolabelled compound was calculated on the basis of the specific activity as stated by the supplier.

Isolation of DNA. Liver DNA was isolated according to Markov & Ivanov (1974) with minor modifications as described in Viviani & Lutz (1978). Liver homogenate was deproteinated with a mixture of chloroform (480 ml), isoamyl alcohol (20 ml) and phenol (added with stirring to give a volume of 1 litre), and the DNA was purified by adsorption chromatography on hydroxyapatite. After dialysis against deionized water and precipitation with ethanol, the DNA was dissolved in 0.014 M-phosphate buffer. The specific activity of the DNA was determined by counting the ^{14}C activity in 10 ml Insta-Gel and by quantitative spectrophotometric determination of the DNA at 260 nm (absorbance was 20 for a solution of 1 mg DNA/ml buffer).

Purine-base analysis. DNA was hydrolysed for 1 hr at 70°C with 0.1 N-HCl to liberate the purines (Lawley, 1976). 7-Methylguanine was added as a standard (Sigma Chemical Co., St Louis, MO) and the mixture (about 100 μg hydrolysed DNA) was separated by reverse-phase HPLC on a Packard 1084 chromatograph with a μBondapak C_{18} column, 300 × 4 mm (Waters Associates, Milford, MA). Elution with a 0.01 M-ammonium phosphate buffer, pH 4, containing 1% methanol at a flow rate of 1.2 ml/min was monitored spectrophotometrically at 260 nm. Fractions of 1.2 ml were collected and ^{14}C radioactivity was counted.

Statistics. To assess the statistical significance of effects, the probability of equality was calculated on the basis of a one-sided Student's *t* test, corrected, if necessary, for inequality of the variance.

RESULTS

Radioactivity on the DNA of an animal treated with a radiolabelled substance is not necessarily due to covalent interactions of the test compound with DNA. Besides non-covalent interactions with DNA or contamination of the DNA with RNA or proteins, it is also possible that biosynthetic incorporation of radioactivity will occur if the radiolabelled compound is degradable to small molecules able to enter the pool of nucleic acid precursors. Such is obviously the case with single-carbon fragments generated in the metabolism of DMA and NDMA, and it is therefore not surprising that liver DNA was found to be radiolabelled even when DMA was administered without nitrite (see Tables 1 and 2 for rat and mouse, respectively). The specific activity of these DNA samples (4–50 dpm/mg) was, however, not high enough to allow for a purine-base HPLC analysis to determine what fraction of the total DNA radioactivity was incorporated into natural purines or was eluting as 7-methylguanine.

In experiment 2, a roughly fifty-fold molar excess of potassium nitrite was administered simultaneously with DMA. The radioactivity in whole liver DNA in all animals was higher than in experiment 1. This increase in radioactivity in whole DNA is no proof on its own of the generation of a methylating agent. It is also possible that this treatment stimulated DNA synthesis and thereby increased the level of biosynthetic incorporation of radiolabelled fragments. Thus it is necessary to distinguish between the various sources of radioactivity. Analysis of the purine bases by HPLC of a number of DNA samples revealed the presence of 7-methylguanine (see tables). This methylated DNA base represented 70–92% of the total radioactivity eluted so it is clear that the administration of nitrite was, in both animal species, responsible for the formation of a DNA-methylating agent.

Experiment 2 also illustrates the very different responses of individual animals, the highest value in the mouse, for example, being nearly six times the lowest value. Some important modulatory factors may be the pH of the stomach, the degree of satiation, and diurnal fluctuations in the rate of DNA synthesis (Schulte-Hermann & Landgraf, 1974), among others. Although this brings great uncertainty to the quantitative evaluation of the data, we tried to correlate the amount of 7-methylguanine formed in experiment 2 with the amount of 7-methylguanine generated after administration of authentic *N*-nitroso[^{14}C]dimethylamine, the methylating agent produced from DMA and nitrite. Experiment 5 (Tables 1 and 2) shows the

Table 1. Formation of 7-methylguanine in male rat liver DNA, 6 hr after oral administration of [^{14}C]dimethylamine.HCl, potassium nitrite and vitamin C

Experiment no.	Treatment (mg/kg body weight)				Results	
	[^{14}C]DMA.HCl	KNO_2	Vitamin C	[^{14}C]NDMA	Specific activity of DNA per unit dose*	% of radioactivity in 7-MG
1	0.70	—	—	—	4.8	ND
	0.33	—	—	—	2.2	ND
Mean ± 1 SD					3.5 ± 1.8	
2	1.15	50	—	—	17.5	70
	1.08	50	—	—	12.8	ND
	1.12	50	—	—	7.6	88
Mean ± 1 SD					12.6 ± 5.0	
3	1.01	50	50	—	4.5	ND
5 (+ve control)	—	—	—	0.48†	1180	ND
	—	—	—	0.28‡	1150	>83
Mean ± 1 SD					1165 ± 21	

NDMA = *N*-Nitrosodimethylamine 7-MG = 7-Methylguanine ND = Not determined; insufficient radioactivity for HPLC analysis of purine bases

*Specific activity is expressed as (dpm/mg isolated DNA)/(dpm/kg body weight) and the values in the table are the true values multiplied by 10^8.

†1.08×10^8 dpm/kg body weight.

‡6.20×10^7 dpm/kg body weight.

results of ^{14}C-labelled NDMA administration: liver DNA was highly radiolabelled and the base analysis revealed that this was predominantly due to methylations and not to biosynthetic incorporation. With these values taken as equivalent to 100% DMA nitrosation, we estimated what percentage of DMA had been converted to NDMA under the conditions used in experiment 2 with simultaneous administration of nitrite. We assumed that the dose-binding relationship was linear down to the small amounts of NDMA generated in our experiments. Good data are available to show that this is indeed the case for the gener-

Table 2. Formation of 7-methylguanine in male mouse liver DNA, 6 hr after oral administration of [^{14}C]dimethylamine.HCl, potassium nitrite and vitamin C or vitamin E

Experiment no.	Treatment (mg/kg body weight)					Results	
	[^{14}C]DMA.HCl	KNO_2	Vitamin C	Vitamin E	[^{14}C]NDMA	Specific activity of DNA per unit dose*	% of radioactivity in 7-MG
1	0.62	—	—	—	—	0.5	ND
2	0.66	50	—	—	—	1.4	ND
	0.69	50	—	—	—	7.7	92
	0.92	50	—	—	—	1.5	ND
	0.98	50	—	—	—	2.6	ND
Mean ± 1 SD						3.3 ± 3	
3	0.66	50	50	—	—	0.9	ND
	0.56	50	50	—	—	0.8	ND
	0.98	50	50	—	—	1.6	ND
	0.98	50	50	—	—	0.8	ND
Mean ± 1 SD						1.0 ± 0.4	
4	0.63	50	—	50	—	1.7	ND
	0.68	50	—	50	—	0.6	ND
	0.88	50	—	50	—	3.4	ND
	0.98	50	—	50	—	1.8	ND
Mean ± 1 SD						1.9 ± 1.2	
5(+ve control)	—	—	—	—	4.77†	369	>99
					1.16‡	382	>95
Mean ± 1 SD						376 ± 9	

NDMA = *N*-nitrosodimethylamine 7-MG = 7-Methylguanine ND = Not determined; insufficient radioactivity for HPLC analysis of purine bases

*Specific activity is expressed as (dpm/mg isolated DNA)/(dpm/kg body weight) and the values in the table are the true values multiplied by 10^8.

†1.07×10^9 dpm/kg body weight.

‡2.61×10^8 dpm/kg body weight.

ation of 7-methylguanine from doses of NDMA as little as 10 μg/kg body weight (Diaz Gomez, Swann & Magee, 1977). It is therefore possible to estimate that, in the rat (Table 1), (12.6–3.5)(100/1165) = 0.78% of the DMA had been nitrosated to yield NDMA. Due to the large individual variations, the extent of nitrosation ranged from 0.2 to 1.3%. The corresponding values in the mouse (Table 2) span 0.2–1.9% with an average of 0.75%, i.e. almost identical to the fraction determined in the rat.

Experiments 3 and 4 elucidated the inhibitory action of vitamin C and E, respectively, on the nitrosation of DMA by nitrite. Because of the similarities in the above results for the rat and the mouse, replicate studies were performed only in the mouse (Table 2). Vitamin C exerted a strong inhibitory effect on the generation of NDMA. After correction of the data for biosynthetic incorporation of radioactivity, it is estimated that 50 mg/kg body weight of sodium ascorbate reduced the formation of NDMA from DMA and nitrite to roughly 20% of the uninhibited level. However, because of the large individual variations, this inhibition was not statistically significant ($P > 0.1$). An equal dose of α-tocopherol acetate reduced the formation of NDMA to about 50% of the uninhibited value, but large individual fluctuations again rendered this result statistically insignificant ($P > 0.2$). In the one rat given vitamin C (Table 1), there was an apparent reduction of DNA radioactivity to background values.

DISCUSSION

The rate of formation of nitrosamines *in vitro* at a given pH is proportional to the concentration of the amine and to the second power of the concentration of nitrite (Mirvish, 1975). The reaction constant, k, is a function of the pH of the reaction mixture and of the nature of the amine. For DMA, the optimum pH is 3.4, and the nitrosation reaction follows the equation

$$d[NDMA]/dt = k[DMA][NO_2^-]^2$$

where k (pH 3.4) = 0.0017 $M^{-2}\,sec^{-1}$.

In our experiments, about 0.8% of the dose of DMA has been converted to NDMA. If we apply the above equation to the present experiment *in vivo* and assume a constant rate of nitrosation, we could determine the time that was necessary for this amount of NDMA to be formed. Assuming a 1 ml reaction volume and a pH of 3.4 in the stomach, and taking the dosage used in the second rat in experiment 2 (weight 220 g) as an example, we find that the reaction time, t, would be given by

$$(2.3 \times 10^{-5})/t = 0.0017(2.9 \times 10^{-3})(1.3 \times 10^{-1})^2$$

thus t = 276 sec. Therefore, a reaction time of about 5 min was required. This seems reasonable considering that the half-life of nitrite in the rat stomach is of the order of 10 min to 1 hr (Mirvish, Patil, Ghadirian & Kommineni, 1975). We therefore conclude that the situation *in vivo* in our experiments does not differ greatly from an *in vitro* incubation reaction. This finding contrasts with the results of Rounbehler *et al.* (1977) who analysed entire frozen mice for NDMA, 40 sec to 60 min after oral gavage of 50 ng DMA and 250 ng nitrite, and recovered up to 14 ng NDMA. They calculated that this amount is 140 times greater than would be expected from an *in vitro* incubation and concluded that model studies *in vitro* cannot take into account the many possible competing processes *in vivo*, including the modifying effects of inhibitors and catalysts.

Recent reports on the formation of nitrosoproline in rats and humans from dietary proline and nitrite or nitrate are consistent with our finding that *in vitro* reaction kinetics are not markedly distorted *in vivo* (Ohshima & Bartsch, 1981; Ohshima, Bereziat & Bartsch, 1982). The similarity of the results for rats and mice in our experiments also suggests that species differences might not be so large as to prohibit the use of animal studies for risk assessments in humans. On the other hand, there were large differences between individual animals. It is to be expected that this finding will be even more pronounced in human populations. The use of average figures may therefore be misleading and it will be necessary, in the long run, to get more information on modulatory factors governing the many steps between the uptake of the reactants and the alkylation of DNA. Such knowledge should then also allow us to define high-risk populations and situations.

With all these limitations in mind, we can tentatively estimate the extent of liver DNA methylations in man from environmental exposure to DMA and nitrite. The dietary uptake of DMA per meal is of the order of 1 mg (Neurath, Dünger, Pein *et al.* 1977) and the concentration of nitrite in the stomach has been reported to range from less than 2×10^{-5} M to more than 2×10^{-3} M, depending primarily on the nitrate content in the diet (Tannenbaum, 1980). If we adopt an average of 10^{-4} M-nitrite, a reaction time of 30 min (1800 sec) and an optimum pH of 3.4, the concentration of NDMA generated is

$$[NDMA] = 1800 \times 0.0017(2 \times 10^{-5})(10^{-4})^2\ M$$

which is about 6×10^{-13} M. Thus, as little as 22 pg NDMA are formed in 500 ml stomach contents. This is not very much compared with the daily intake in man of preformed dietary nitrosamines, i.e. about 0.5 μg NDMA (Spiegelhalder, Eisenbrand & Preussmann, 1980), so the amount of NDMA formed *in vivo* in the stomach seems to be negligible. The concentrations of DMA and nitrite reached in the stomachs of the animals we studied are higher than our estimates of human concentrations by a factor of at least 100 and 1000, respectively. The concentration of NDMA formed was therefore about 10^8 times higher in our experiment compared with the normal situation in man. Our system is not sensitive enough to be used with much lower concentrations. For greater sensitivity it would be necessary to use an amine with a higher reaction constant, k, such as aminopyrine, which readily generates NDMA on reaction with nitrite. We are currently investigating this reaction with lower concentrations of nitrite and studying the conversion of nitrate to nitrite.

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