Methylation of DNA by incubation with methylamine and nitrite

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DNA was incubated in septum-closed reaction vials with [14C]methylamine and nitrite. The DNA was purified, hydrolysed with hydrochloric acid, and the purines were analysed by h.p.l.c. 7-Methylguanine was detectable as a result of DNA methylation in experiments performed in 100 mM acetate at pH 4. Using different concentrations of amine and nitrite a first order reaction for total amine and a second order for total nitrite could be shown. A study on the pH dependence using 100 mM malonate buffer, pH 2.0—6.0, revealed a maximum rate at pH 3.5, with steep slopes above and below this pH value, in agreement with a mathematical analysis of the reaction equations. The data show that the alkylating agent formed spontaneously by nitrosation and deamination of a *primary* amine has a long enough lifetime to react with DNA *in vitro*. Using the reaction orders established here, an extrapolation to lower concentrations found in the stomach can now be performed. Future *in vivo* experiments on the methylation of gastro-intestinal DNA then would show to what extent DNA in a cell is protected from alkylation.

Introduction

The generation of nitrosamines from amines and nitrite under acidic conditions could represent an important mechanism for the formation of carcinogens in the stomach (1) from ubiquitous dietary amines (2) and unavoidable salivary nitrite (3,4). With primary amines, chemically unstable products are formed which react readily with nucleophiles. This instability must have been the reason for the negligible interest of toxicologists in primary amines as opposed to the secondary amines known to produce chemically stable nitrosamines.

Nevertheless it seemed important to investigate a DNA damage exerted by the nitrosation products of methylamine, a dietary constituent of fish (5) and vegetables (2). Since the chemistry predicted the formation of a methylating agent (6), methylated DNA bases were searched using techniques established in earlier work with dimethylamine (7). Establishing dose dependences both for the amine and for nitrite and investigating the pH dependence seemed to be indicated because preliminary experiments using [14C]methylamine allowed the detection of DNA methylations (8). The results reported here allowed us to form an equation for the reaction kinetics at different pH values, information which will ultimately be required for an extrapolation to the lower concentrations encountered in a human stomach.

Materials and methods

Chemicals and apparatus

L(+)-Sodium ascorbate and potassium nitrate were obtained from Fluka AG

(Buchs, Switzerland). DNA from calf thymus (sodium salt type I; highly polymerized) and 7-methylguanine were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were purchased in the highest purity available from Merck (Darmstadt, FRG). [14C]Methylamine hydrochloride (mol. wt. 67.5) with a specific activity of 50 mCi/mmol, dissolved in ethanol (100 μCi/ml), was obtained from New England Nuclear (Boston, MA). The radiochemical purity was 99%, as determined by t.l.c. on cellulose (Merck, Darmstadt, FRG) using methanol:diethylether:1N hydrochloric acid:water (10+10+1+3) as the solvent system. The ethanol from the original [14C]-methylamine hydrochloride solution was evaporated with nitrogen and the salt redissolved in incubation buffer to give a stock solution with a specific radioactivity of 245 μCi/ml. Dialysis tubing (Visking Type 20/32, mol. wt. exclusion at 12000—14000 daltons; diameter 17 mm) was obtained from Union Carbide (Chicago, IL).

Radioactivity measurements were carried out in 10 ml of Insta-Gel (Packard Instruments, Downers Grove, IL) in a liquid scintillation counter, model Packard Tri Carb 460 CD. The h.p.l.c. analysis of the DNA bases was performed on a semipreparative μBondapak C_{18} column, 300 x 7.8 mm (Waters Associates, Millford, MA) equipped with two h.p.l.c. pumps (model LC Pump 410 from Kontron, Zürich, Switzerland) controlled by a Kontron Programmer 200 to generate a linear gradient of two eluents.

General methods

Incubation system. Stock solutions of unlabelled methylamine, [14C]methylamine, nitrite, nitrate and DNA were prepared in 100 mM potassium acetate/acetic acid pH 4 or 100 mM malonic acid/sodium hydroxide or hydrochloric acid (pH dependence). Appropriate volumes of amine and DNA (where applicable) were mixed to give the concentrations wanted in a final volume of 10 ml in 24.6 ml glass vials tightly closed with a septum. The reaction was started by injection of potassium nitrite dissolved in the same buffer. The solution was kept at 37°C, slowly stirred with a magnetic stirrer, and the reaction was stopped after 30 min by addition of sodium ascorbate in 2-fold molar excess of the nitrite concentration (9). Immediately before stopping the reaction, a 1 ml sample of the gas phase above the reaction mixture was taken out with a syringe and injected into a 2 ml serum vial prefilled with 1 g methanol and closed with a septum. After thorough shaking, 1 ml of this methanol solution was pulled up into a syringe and mixed into 10 ml Insta-Gel for scintillation counting.

Isolation of DNA. The incubation buffer was dialysed three times at 4°C against 10 l 0.2 M NaCl for 5—10 h in order to remove most of the non-covalently bound radioactive species. The DNA was precipitated by adding 2 volumes ethanol and storing at −20°C overnight. After centrifugation for 20 min at 1000 g the supernatant was decanted and the DNA was dried *in vacuo* for 2—3 h. The DNA was dissolved in 10 mM MgCl_2, 10 mM Tris/HCl, pH 7.0 and reprecipitated once with ethanol. The amount of DNA was determined by u.v. spectroscopy using as standard an absorbance of 20 at 260 nm for a solution of 1 mg DNA per ml.

Depurination of DNA and h.p.l.c. analysis. DNA was hydrolysed for 1 h at 70°C with 0.1 N HCl to liberate the purines (10). 7-Methylguanine was added as standard, the mixture was filtered through a 0.2 μm filter (Millipore, Bedford, MA) and loaded on a reverse-phase h.p.l.c. system. Elution medium was a 10 mM ammonium phosphate buffer, pH 4.0, containing methanol (1% for 20 min, followed by a linear gradient to 100% methanol in 40 min). The flow rate was 3.5 ml/min and the optical density of the eluate was recorded at 260 nm. Fractions of 2 min were collected and the radioactivity of each fraction was determined by scintillation counting. To avoid phase separation between the scintillation cocktail and the eluate, fractions 19, 20 and 21 were diluted with 1 g water, 1 g methanol and 0.5 g methanol, respectively. The retention times of the purines, guanine, adenine and 7-methylguanine were 10.2, 14.8 and 20.4 min, respectively. The recovery of the injected radioactivity ranged from 90 to 110%. To compare the results of the different experiments, a 7-methylguanine index was defined as micromol 7-methylguanine/mol DNA nucleotide.

Time dependence of the deamination. Three experiments were performed with 2.5 mM, 1.5 mM, 3.0 mM [14C]methylamine hydrochloride (sp. act. 50 μCi/mmol) in 100 mM acetate buffer pH 4.0, incubated with 78.8 mM, 78.8 mM and 50.4 mM potassium nitrite, respectively. The control incuba-

tion (3.0 mM [¹⁴C]methylamine hydrochloride) was 74.2 mM in potassium nitrate. The radioactivity in the gas phase was determined at different times up to 6 h.

Nitrite concentration dependence. A solution of 1.2 mM [¹⁴C]methylamine hydrochloride (sp. act. 2.56 mCi/mmol) and 1.05 mg/ml calf thymus DNA (equivalent to 3.4 mM in nucleotides) was incubated with 0.0, 4.4, 13.0, 22.0, 44.0 and 66.0 mM sodium nitrite. In order to reach a constant ionic strength in the incubations, potassium nitrate was added to give a total concentration of 66 mM for nitrite plus nitrate.

Amine concentration dependence. Solutions of 15 μM, 45 μM, 0.14 mM, 0.38 mM and 1.2 mM [¹⁴C]methylamine hydrochloride (sp. act. 58.6, 58.6, 6.31, 6.31, 0.68 mCi/mmol, respectively) were incubated with 66 mM sodium nitrite in the presence of 1.02 mg/ml calf thymus DNA. The control contained 1.2 mM [¹⁴C]methylamine hydrochloride (sp. act. 0.68 mCi/mmol), 3.3 mM DNA nucleotide and 82 mM potassium nitrate.

pH dependence. The reaction vials contained 1 mM [¹⁴C]methylamine hydrochloride (sp. act. 0.58 mCi/mmol) in 100 mM malonate buffer at the following pH values: 2.0, 2.6, 3.3, 3.7, 4.6, 5.0, 6.0. Sodium nitrite was added to a final concentration of 70 mM to start the deamination reaction. Two controls with 66 mM potassium nitrate instead of nitrite were run at pH 1.5 and 5.1. No change in pH was noticed during the 30 min incubation period.

Results

Time-dependence of the deamination of methylamine

The reaction of [¹⁴C]methylamine with nitrite is known to yield [¹⁴C]methanol and [¹⁴C]methyl nitrite (11), two volatile products expected to appear in the gas phase above the reaction mixture. The simple determination of the radioactivity in the headspace could therefore be used to follow the appearance of the reaction products in order to assess the rate of the formation of electrophilic intermediates such as the methyl diazonium ion. Figure 1 shows a linear time-dependence up to ~1 h using three different sets of concentrations of methylamine and nitrite. For the following studies, incubation times of 30 min were therefore chosen to make sure that no changes of the reaction kinetics by side reactions would confuse the analysis of the data. The control experiment with nitrate instead of nitrite (open circles in Figure 1) showed that the incubation of [¹⁴C]methylamine with nitrate did not lead to the generation of volatile radio-labelled reaction products.
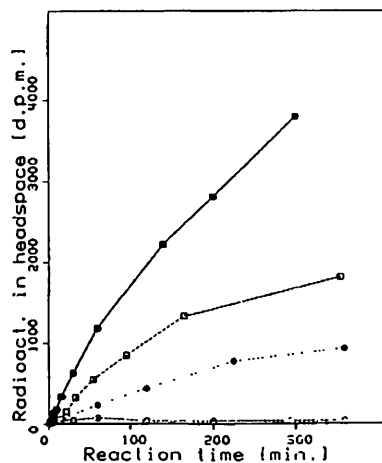
In vitro methylation of DNA

Nitrite concentration dependence. Figure 2 shows the h.p.l.c. radioactivity elution profile of depurinated DNA after incubation with [¹⁴C]methylamine and different concentrations of potassium nitrite. The top chart is the control DNA digest obtained when using nitrate in the place of nitrite. Sixty to seventy percent of the radioactivity always coeluted with
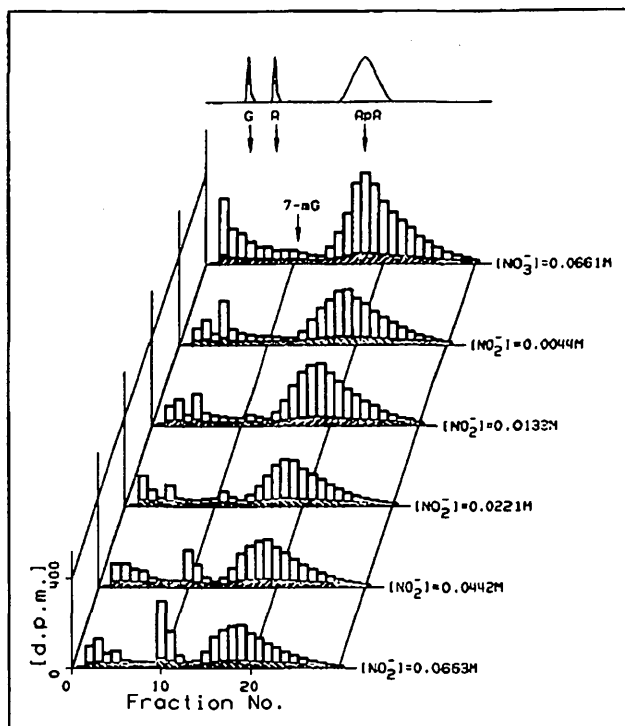
Fig. 2. H.p.l.c. elution profile of acid-hydrolysed DNA isolated 30 min after incubation with 1.2 mM [¹⁴C]methylamine and different concentrations of nitrite. Separate top chart: optical density profile at 260 nm, showing elution region for guanine (G), adenine (A), 7-methylguanine (7-mG) and apurinic acid (ApA). Top radioactivity profile: control incubation with nitrate instead of the highest concentration of nitrite.
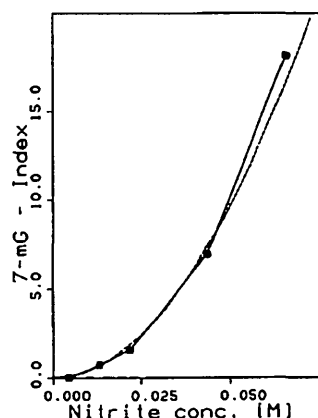
Fig. 1. Rate of formation of volatile reaction products determined in the closed air volume above a solution of [¹⁴C]methylamine hydrochloride and potassium nitrite in a 100 mM acetate buffer, pH 4.0. The concentrations of amine and nitrite, respectively, were: ■——■, 0.0025 M, 0.0788 M; □- - -□, 0.0015 M, 0.0788 M; ●····●, 0.003 M, 0.0504 M; O–··–O, control incubation with 0.003 M [¹⁴C]methylamine hydrochloride and 0.072 M potassium nitrate.

Fig. 3. Formation of 7-methylguanine in DNA incubated for 30 min in 0.1 M acetate buffer pH 4.0, containing 1.2 mM [¹⁴C]methylamine and different concentrations of potassium nitrite. The ionic strength of all solutions was made equal by appropriate additions of potassium nitrate. ■——■, experimental data; ——, theoretical dose dependence obtained by using the equation 7-mG-Index = k' x [total amine] x [total nitrite]² with k' = 4.0 x 10⁶ M⁻³ (see Discussion section).

the apurinic acid, probably representing DNA phosphate-alkylammonium salts. At the retention of 7-methylguanine (fractions 10 and 11), increasing amounts of radioactivity appeared with higher nitrite concentrations. Net counts in the fractions 10 and 11 were determined by subtraction of a background calculated as the mean radioactivity of the fractions 9 and 12. The control DNA digest as well as the incubation with the lowest concentration of nitrite contained no 7-methylguanine. The remaining counts were spread over the front fractions, the region known to contain guanine (fraction 5) and adenine (fractions 7 and 8). Upon conversion of the net radioactivity in fractions 10 plus 11 to methyl groups on the basis of the known specific activity of the methylamine used and the amount of DNA hydrolysed, a 7-methylguanine index (= μmol 7-methylguanine/mol DNA nucleotide) was defined and plotted in Figure 3 against the nitrite concentration. The dashed line which was calculated according to the square of the nitrite concentration nicely overlaps with the experimental points and is a clear indicator of a second order reaction of nitrite with methylamine to produce DNA methylations.

Amine concentration dependence. Using the highest nitrite concentration taken above and varying the amine concentration, an analogous experiment was performed. Figure 4 clearly shows the linear dependence of the DNA methylations on the amine concentration.

pH dependence. In both experiments described above, the headspace radioactivity was determined at the end of the 30-min incubation period, before the quantification of DNA methylations. Figure 5 shows the linear relationship between these two experimental endpoints. Since headspace activities can be determined using relatively little radioactivity, these values were taken for the determination of the pH dependence of the nitrosation reaction. The proportionality to the DNA methylations makes it possible to use headspace data obtained from short incubation times as a substitute for the 7-methylguanine-index. Figure 6A clearly shows that the generation of volatile [^{14}C]methyl derivatives after incubation of [^{14}C]methylamine and nitrite has a maximum value at a pH of ~3.5 with very steep slopes above and below.

Discussion

We have shown that the rate of formation of a DNA-methylating agent by incubation of methylamine with DNA in the presence of nitrite follows the equation

d[7-methylguanine]/dt = k x [total amine] x [total nitrite]^2

Using the present data on the formation of 7-methylguanine in our standard incubation system, the equation reads

7-methylguanine index = k' x [total amine] x [total nitrite]^2

with k' = 4.0 x 10^6 M^{-3} determined as an average of all single experimental values. The reaction order, therefore, is in accordance with the mechanism of nitrosation of secondary
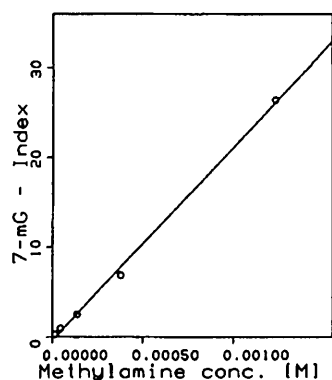

Fig. 4. Formation of 7-methylguanine in DNA incubated for 30 min in 0.1 M acetate buffer pH 4.0, containing 66.0 mM potassium nitrite and different concentrations of [^{14}C]methylamine. Correlation coefficient for a linear regression analysis: R = 0.999.
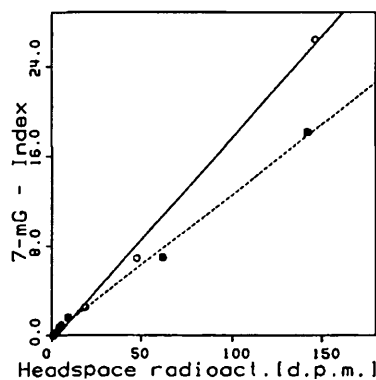

Fig. 5. Correlation of the methylation of DNA with the formation of volatile radiolabelled products. O——O, data from the dose dependence for methylamine, R = 0.998; ■——■, data from the dose dependence for nitrite, R = 0.998.
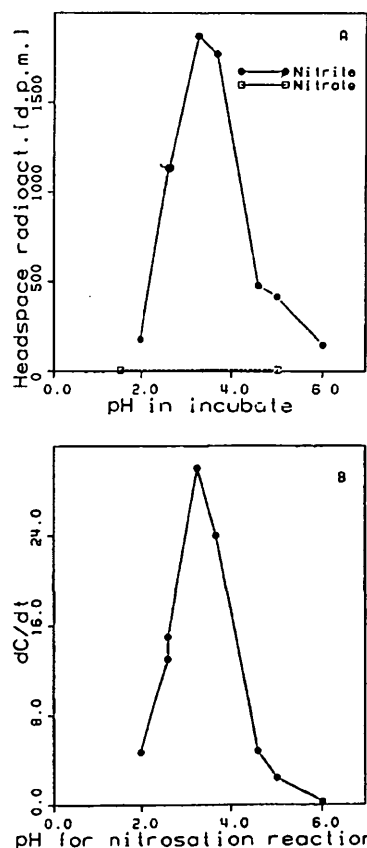

Fig. 6. Formation of volatile radiolabelled reaction products at different pH values. A, Experimental values; B, Curve calculated on the basis of equation (2).

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amines elucidated by Ridd (6) and Mirvish (12). The reactions which can take place in our incubation system are compiled in a review by Douglass *et al.* (13) where he discusses the different nitrosating agents. The fact that the reaction is second order in nitrite is an indication favoring N_2O_3 as the most important nitrosating agent in our system. Since N_2O_3 can be regarded as the anhydride of two nitrous acid molecules it is obvious that its concentration increases with decreasing pH.

The amine reactant must be in the neutral form to be nitrosatable. The ratio of neutral to protonated form is dependent on the pH and the basicity of the amine. The lower the pH, the less nitrosatable amine is available. The situation is therefore characterized by the following reaction kinetics

$$dC/dt = k'' \times [A] \times [HNO_2]^2 \tag{1}$$

The concentrations of the reactive species [A] and [HNO_2] in solution can be calculated in terms of the total amine [A]$_{tot}$ and total nitrite [NO_2^-]$_{tot}$ concentration, given that the latter values are equal to the sum of the protonated and the deprotonated forms for each component. Substitution into the respective acid dissociation equilibria and rearrangement yields:

$$\frac{dC}{dt} = k'' \times [A]_{tot} \times [NO_2^-]_{tot}^2 \times \frac{K_1 \times [H^+]^3}{[H^+]^3 + (K_1 + 2K_3)\times[H^+]^2 + (K_3^2 + 2K_1K_3)\times[H^+] + K_1K_3^2} \tag{2}$$

Using $K_1 = 2.3 \times 10^{-11}$ and $K_2 = 4.3 \times 10^{-4}$ for the acidity of methylammonium ion and of nitrous acid, respectively, the graphical representation of this equation in terms of the reaction rate as a function of the pH is shown in Figure 6B. Shape and position of the maximum are almost identical to the experimentally observed data (Figure 6A).

7-Methylguanine is the most abundant methylated base formed upon reaction of DNA with methylating carcinogens, representing ~80% of all DNA methylations (10), and was the only methylation product detectable in the present assay. The level of DNA methylation in the 7-position of guanine is not correlated with mutagenic or carcinogenic effects. Other methylation products, such as O^6-methylguanine, seem to be more important. Nevertheless, the present data are interpretable in terms of the formation of this promutagenic lesion because the ratio between 7- and O^6-methylations is known to be 10 to 1 for methyl diazonium ion, the ultimate methylating agent, well known from dimethylnitrosamine studies (10) and expected to be formed here, too.

Methylation of DNA by methylamine and nitrite has already been reported two decades ago, but this was in the chemical context of DNA derivatization by diazomethane (14). The toxicological aspect so far has received little attention (15) as compared with the formation of carcinogenic nitrosamines from secondary amines (16). These latter compounds are chemically stable and are converted to reactive derivatives only upon oxidative enzymatic dealkylation. DNA alkylation is therefore expected primarily in cells which contain the appropriate enzyme systems. The nitrosation of primary amines is known to the chemist to yield highly unstable intermediates which spontaneously react with water to yield the corresponding alkyl alcohols. This rapid inactivation at the site of formation was probably the reason why biologists did not investigate the potential DNA alkylating activity. Indeed, the extent of DNA methylations was very low (of the order of 0.01% with respect to methylamine) in our *in vitro* system, even using high concentrations of nitrite and optimum pH, and incubating the DNA *in situ*. Sporadic reports have shown, otherwise, that a mutagenicity to micro-

organisms can be generated by incubation with primary amines and nitrite (17,18). In addition, we have reported preliminary data on a DNA methylation of gastro-intestinal DNA in rats that had been administered [^{14}C]methylamine and nitrite (8). All these experiments have been performed using a nitrite concentration which was 2−3 orders of magnitude higher than the one expected to be encountered in a stomach. More refined *in vivo* work will therefore be required before the role of primary amines in the etiology of cancer can be assessed.

Acknowledgement

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