Carcinogenesis vol.25 no.8 pp.1525–1533, 2004
doi:10.1093/carcin/bgh156

Syntheses of DNA adducts of two heterocyclic amines, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) and identification of DNA adducts in organs from rats dosed with MeAαC

Hanne Frederiksen[2], Henrik Frandsen and Wolfgang Pfau[1]

Institute of Food Safety and Nutrition, Danish Veterinary and Food Administration, Mørkhøj Bygade 19, DK 2860 Søborg, Denmark and [1]Institute of Clinical and Experimental Toxicology, Hamburg University Medical School, Vogt-Kolln Strasse 30, D22527 Hamburg, Germany

[2]To whom correspondence should be addressed
Email: haf@fdir.dk

2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (AαC) are mutagenic and carcinogenic heterocyclic amines formed during ordinary cooking. MeAαC and AαC are activated to mutagenic metabolites by cytochrome P450-mediated *N*-oxidation to the corresponding N^2-OH derivatives. The proximate mutagenic N^2-OH derivatives of MeAαC and AαC did not react with deoxynucleosides or DNA. However, upon acetylation with acetic anhydride both reacted with 2′-deoxyguannosine and 3′-phospho-2′-deoxyguanosine, resulting in one adduct each, but not with other nucleosides or nucleotides. The adducts were identified as N^2-(2′-deoxyguanosin-8-yl)-MeAαC, N^2-(2′-deoxyguanosin-8-yl)-AαC, N^2-(3′-phospho-2′-deoxyguanosin-8-yl)-MeAαC and N^2-(3′-phospho-2′-deoxyguanosin-8-yl)-AαC by comparison with adducts of known structure obtained by reaction of the parent amines with acetylated guanine *N*3-oxide. N^2-OH-MeAαC and N^2-OH-AαC reacted with calf thymus DNA after addition of acetic anhydride. ^{32}P-postlabelling analysis of modified DNA showed one major adduct co-migrating with N^2-(3′,5′-diphospho-2′-deoxyguanosin-8-yl)-MeAαC and N^2-(3′,5′-diphospho-2′-deoxyguanosin-8-yl)-AαC, respectively. Some minor adducts presumed to be undigested oligomers were also detected. ^{32}P-postlabelling analysis of DNA from several organs of rats dosed orally with MeAαC showed that *in vivo* N^2-(2′-deoxyguanosin-8-yl)-MeAαC also was the major adduct formed. Relative adduct level in DNA isolated from the liver of the rats was about 50.40 adducts/10^9 nt. The adduct levels were ~4-fold lower in the colon and the heart and ~12-fold lower in the kidney of the rats.

Introduction

2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) and 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) are two food-borne mutagenic and carcinogenic heterocyclic amines (1). MeAαC and AαC are formed as pyrolysis products of either tryptophan or proteins of animal or vegetable origin, e.g. soybean globulin (2,3). MeAαC and AαC are found in cooked food such as fried meat, chicken, fish (4–8) and cigarette smoke condensates (9–11). MeAαC is also found in wine (12). Dietary administration of MeAαC and AαC in rodents has shown that they are moderately potent carcinogens (13–15).

Like other heterocyclic amines, MeAαC and AαC undergo metabolic activation resulting in formation of the corresponding N^2-OH derivatives (16,17). Previously an *in vitro* study has shown that ~50 and 60% of MeAαC and AαC, respectively, were phase I activated in hepatic rat microsomes and in pools of human hepatic microsomes (18). Activated *N*-OH derivatives of heterocyclic amines are usually further activated by, for example, acetylation (19). This results in formation of reactive *N*-acetoxy derivatives, which spontaneously undergo heterolytic fission into electrophilic arylnitrenium ion intermediates, which are able to form adducts with macromolecules such as proteins and DNA (20). The specific mutagenic activity of MeAαC and AαC in the *Salmonella* assay are low compared with the aminoimidazoazaarenes (21,22), but DNA binding studies showed higher covalent modification of liver DNA from rats treated with MeAαC and AαC than from rats treated with 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]-pyridine (PhIP) (23,24). ^{32}P-postlabelling analysis of DNA modified with both MeAαC and AαC *in vitro* (25,26) and *in vivo* (14,24) showed one major DNA adduct and several minor adducts. The major adducts were identified as N^2-(2′-deoxyguanosin-8-yl)-AαC (dG-AαC) and N^2-(2′-deoxyguanosin-8-yl)-MeAαC (dG-MeAαC) by comparison with products obtained by reaction of the parent amine and acetylated guanine *N*3-oxide (25,26).

The aim of the present work was to synthesize N^2-deoxynucleoside-MeAαC and N^2-deoxynucleoside-AαC, usable as standards in ^{32}P-labeling analyses of DNA adducts and compare them with *in vitro* and *in vivo* modified DNA and also to investigate whether activated N^2-OH compounds form adducts other than guanin-8-yl adducts. The method used was acetylation of N^2-OH compounds followed by covalent binding to 2′-deoxynucleosides, according to the previously described synthesis of N^2-(2′-deoxyguanosin-8-yl)-PhIP (27).

Abbreviations: AαC, 2-amino-9H-pyrido[2,3-*b*]indole; dG-AαC, N2-(2′-deoxyguanosin-8-yl)-AαC; dG-MeAαC, N2-(2′-deoxyguanosin-8-yl)-MeAαC; 4,8-DiMeIQx, 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]quinoxaline; G-AαC, N2-(guanin-8-yl)-AαC; G-MeAαC, N2-(guanin-8-yl)-MeAαC; IQ, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline; MeAαC, 2-amino-3-methyl-9H-pyrido[2,3-*b*]indole; MeIQx, 2-amino-4,8-dimethylimidazo[4,5-*f*]quinoxaline; P-dG-AαC, N2-(3′-phospho-2′-deoxyguanosin-8-yl)-AαC; P-dG-MeAαC, N^2-(3′-phospho-2′-deoxyguanosin-8-yl)-MeAαC; PhIP, 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine; RAL, relative adduct level.

Materials and methods

Chemicals

MeAαC and AαC were obtained from Toronto Research Chemicals (Ontario, Canada). Tritiation of MeAαC ([^{3}H]MeAαC) was described previously (16). N^2-OH-MeAαC and N^2-OH-AαC were prepared by chemical synthesis as previously described (18). 2′-Deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine and 2′-deoxythymidine, guanine *N*3-oxide, calf thymus DNA, nuclease P1, alkaline phosphatase, RNase T1 and micrococcal endonuclease (from *Staphylococcus aureus*) were obtained from Sigma (St Louis, MO). ATP and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Hoffmann-La Roche (Basel, Switzerland) and Amersham (Braunschweig, Germany), respectively.

Phosphodiesterase (from calf spleen) were obtained from Calbiochem (Darmstadt, Germany). Bicine, dithiothretiol and proteinase K were obtained from Merck (Darmstadt, Germany). RNase A and spermidine were obtained from Serva (Heidelberg, Germany) and polynucleotide kinase from Fermentas (St Leon-Rot, Germany). Isolute 101 columns were obtained from International Sorbent Technology (Glamorgan, UK). PEI–cellulose CEL 300 PE sheets were from Macherey & Nagel (Düren, Germany), HPLC grade acetonitrile and formic acid (distilled before use) were obtained from Romil (Cambridge, UK). Soluene-350 and Hionic-Flour were obtained from Packhard (Meriden, CT). All other chemicals were of analytical grade.

Acetylation of N^2-OH-MeAαC and N^2-OH-AαC and reaction with 2′-deoxynucleosides

The acetylation reaction was conducted under continued fast stirring under argon at −20°C. An aliquot of 275 µl of N^2-OH-AαC or N^2-OH-MeAαC (112 or 149 µg/ml dissolved in dimethyl formamide) was added to 275 µl of ice-cold water, followed by 10 µl of acetic acid. After 3 min, four portions of 3 µl of acetic anhydride were added in intervals of 2 min. The N^2-acetoxy derivatives of N^2-OH-MeAαC and N^2-OH-AαC were prepared immediately prior to reactions with 2′-deoxynucleosides.

2′-Deoxyadenosine, 2′-deoxycytidine, 2′-deoxyguanosine and 2′-deoxythymidine were dissolved at a concentration of 1 mg/ml in 0.1 M sodium phosphate buffer, pH 7.4. Aliquots of 700 µl of each solution and a control not containing 2′-deoxynucleoside were placed in test tubes and kept at room temperature under continued stirring. To each of the tubes was added 80 µl of 50% dimethyl formamide in water. Four portions of 25 µl of the N^2-acetoxy derivatives of N^2-OH-MeAαC or N^2-OH-AαC were added in intervals of 1 min, followed by 10 min stirring at room temperature. pH values in the mixtures were increased to pH 7–7.5 by addition of 1 M sodium phosphate buffer, pH 7.6, before storing at −20°C. For comparison, N^2-(guanin-8-yl)-MeAαC (G-MeAαC) and N^2-(guanin-8-yl)-AαC (G-AαC) were synthesized by reaction of the parent compounds with acetylated guanine *N*3-oxide as previously described (25,26). The reaction mixtures were analysed by HPLC-MS.

Syntheses of N^2-(3′-phospho-2′-deoxyguanosin-8-yl)-MeAαC (P-dG-MeAαC) or N^2-(3′-phospho-2′-deoxyguanosin-8-yl)-AαC (P-dG-AαC)

To 1 ml of 3′-phospho-2′-deoxyguanosine (1 mg/ml dissolved in 0.1 M sodium phosphate buffer, pH 7.4) was added 20 µl of N^2-OH-MeAαC (149 µg/ml dissolved in dimethyl formamide) or N^2-OH-AαC (112 µg/ml dissolved in dimethyl formamide) at room temperature under continued fast stirring under argon. After 3 min, four portions of 3 µl of acetic anhydride was added at intervals of 1 min, followed by a further 10 min stirring. pH values in the mixtures were increased to pH 7–7.5 by addition of 1 M sodium phosphate buffer, pH 7.6, before storing at −20°C. Adducts were analysed by HPLC-MS and ^{32}P-postlabelling analyses.

Stability test

The stability of the adducts dG-MeAαC, dG-AαC, P-dG-MeAαC and P-dG-AαC were investigated by incubation of the adducts in phosphate and acetate buffers with pH values from 5.2 to 7.4 in a shaking water bath at 37°C for 0 to 48 h. Adducts were analysed by HPLC-MS.

Modification of calf thymus DNA with MeAαC and AαC

Calf thymus DNA was covalently modified with MeAαC and AαC in the same manner as the modification of 3′-phospho-2′-deoxyguanosine. The modified calf thymus DNA was precipitated by addition of 30 µl of 3 M sodium acetate and 1.5 ml of ice-cold ethanol. The DNA was isolated by centrifugation and washed with ethanol, followed by drying of the DNA at 30°C under a stream of nitrogen. The modified calf thymus DNA was enzymatically digested by a method modified after Palmgren *et al*. (28). The DNA was dissolved in 0.5 ml of water and 2 ml of 30 mM sodium acetate in 1 mM zinc sulphate, pH 7.3, 20 µl of nuclease P1 and 20 µl of alkaline phosphatase was added, followed by 2 h incubation at 37°C in a shaking water bath. The digest was purified and concentrated on an activated Isolute 101 column (washed before use with 2 ml of methanol followed by 2 ml of water). The column was washed with 1 ml of water and eluted with three portions of 200 µl of 50% dimethyl formamide in methanol. The elutes were analysed by HPLC-MS and ^{32}P-postlabelling analyses.

HPLC-MS analyses

HPLC analyses were performed on a model 1100 liquid chromatograph equipped with a photodiode array detector (Agilent Technologies, Wallbronn, Germany). The adducts were separated on a Zorbax SB-C3, 5 µm, 150 × 3 mm column from Agilent Technologies. Injection volumes were 12.5 µl, with a flow rate of 0.4 ml/min and an oven temperature of 20°C. Solvents were: A, 0.001% formic acid; B, acetonitrile. Solvent programming was: 0–2 min, 10% B; 20 min, 80% B; 22 min, 2% B.

Positive ion electrospray mass spectra were obtained with a MSD 1100 mass spectrometer equipped with an electrospray interface (Agilent Technologies). The following interphase settings were used: nebulizer pressure 60 p.s.i.; drying gas (nitrogen) 10 l/min, 350°C; capillary voltage 4000 V; fragmentor voltage 40 V.

Treatment of animals and isolation of DNA from tissues

Three adult male Wistar rats (aged 7–8 weeks, weight ~200 g) were delivered from Taconic M&B (Lille Skensved, Denmark). After a 5 days acclimatization period, the animals received a single dose of 0.2 ml of [^{3}H]MeAαC (8.6 mg/ml dissolved in 50% dimethyl formamide) by oral gavage. The animals were killed after 3 days and the liver, colon, kidney and heart were removed, washed with saline and stored at −80°C. Recovery of radioactivity was analysed by liquid scintillation counting. A 100 mg tissue sample from each organ was cut in small pieces and added to 1 ml of Soluene-350, mixed and incubated for 2–4 h at 56°C. The tissue homogenates were added to 4 ml of Hionic-Flour and incubated for 3 days in the dark, followed by liquid scintillation counting. Protein-bound radioactivity was analysed by solubilization of 100 mg of each organ in 1 ml of water by sonication. Fifty microlitres of trichloroacetic acid was added and the solution was centrifuged and the supernatant removed. To the pellet was added 200 µl of water and 1 ml of Soluene-350, followed by mixing and incubation for 30 min at room temperature. Finally, the sample was added to 4 ml of Hionic-Flour, followed by liquid scintillation counting. Liquid scintillation counting was performed on a Tri Carb 3100TR with external standardization (Packhard, Meriden, CT).

For ^{32}P-postlabelling analyses DNA was isolated from the liver, colon, kidney and heart of the rats by the phenol extraction method (29) with modifications (30).

^{32}P-postlabelling of (Me)AαC–DNA adducts formed in vitro and in vivo

DNA purified from rat tissues and modified calf thymus DNA were hydrolysed, enriched (butanol enrichment) and ^{32}P-postlabelled by previously described methods (31,32) with modifications according to the standardization and validation of DNA adduct postlabelling methods (33).

The adduct nucleotides were separated by TLC analyses performed by previously described methods (33) with minor modifications (30). Resolution of modified nucleotide sample aliquots was performed by standard two-directional chromatography using a solution of 250 mM ammonium sulphate and 40 mM sodium dihydrogen phosphate as the mobile phase. Quantitation of adduct levels was accomplished by Cerenkov counting of excised adduct spots according to published calculation procedures (34,35). HPLC analysis of ^{32}P-postlabelled adducts was performed according to the method of Pfau *et al*. (36) with modifications according to Wolz *et al*. (30). Relative adduct level (RAL) values were calculated according to Randerath *et al*. (32).

Results

Formation of N^2-acetoxy derivatives and reaction with 2′-deoxynucleosides

Incubation of N^2-OH-MeAαC and N^2-OH-AαC with deoxynucleosides did not result in formation of adducts. However, acetylation of the N^2-OH derivatives to the putative N^2-acetoxy derivatives prior to reaction with 2′-deoxyguanosine resulted in the formation of adducts. The high reactivity of the N^2-acetoxy derivatives prevented chromatographic analysis, because the compound decomposed in the chromatographic system. Therefore, optimum acetylation conditions were determined by analysis of the final product after reaction with 2′-deoxyguanosine. Important parameters in the acetylation reaction were absence of atmospheric oxygen, reaction time and temperature. The highest yield of dG-MeAαC and dG-AαC were obtained by acetylation at −20°C for 8 min in an argon atmosphere, followed by reaction with excess 2′-deoxyguanosine at room temperature.

Under these conditions, addition of the N^2-acetoxy derivates to 2′-deoxyadenosine, 2′-deoxycytidine and 2′-deoxythymidine did not result in formation of detectable amounts of adduct. Figure 1 shows the HPLC profiles of the crude reaction products of the N^2-acetoxy derivates of MeAαC and AαC with 2′-deoxyguanosine. In addition to MeAαC, the chromatogram (Figure 1A) showed two major compounds eluting at 13.4 and

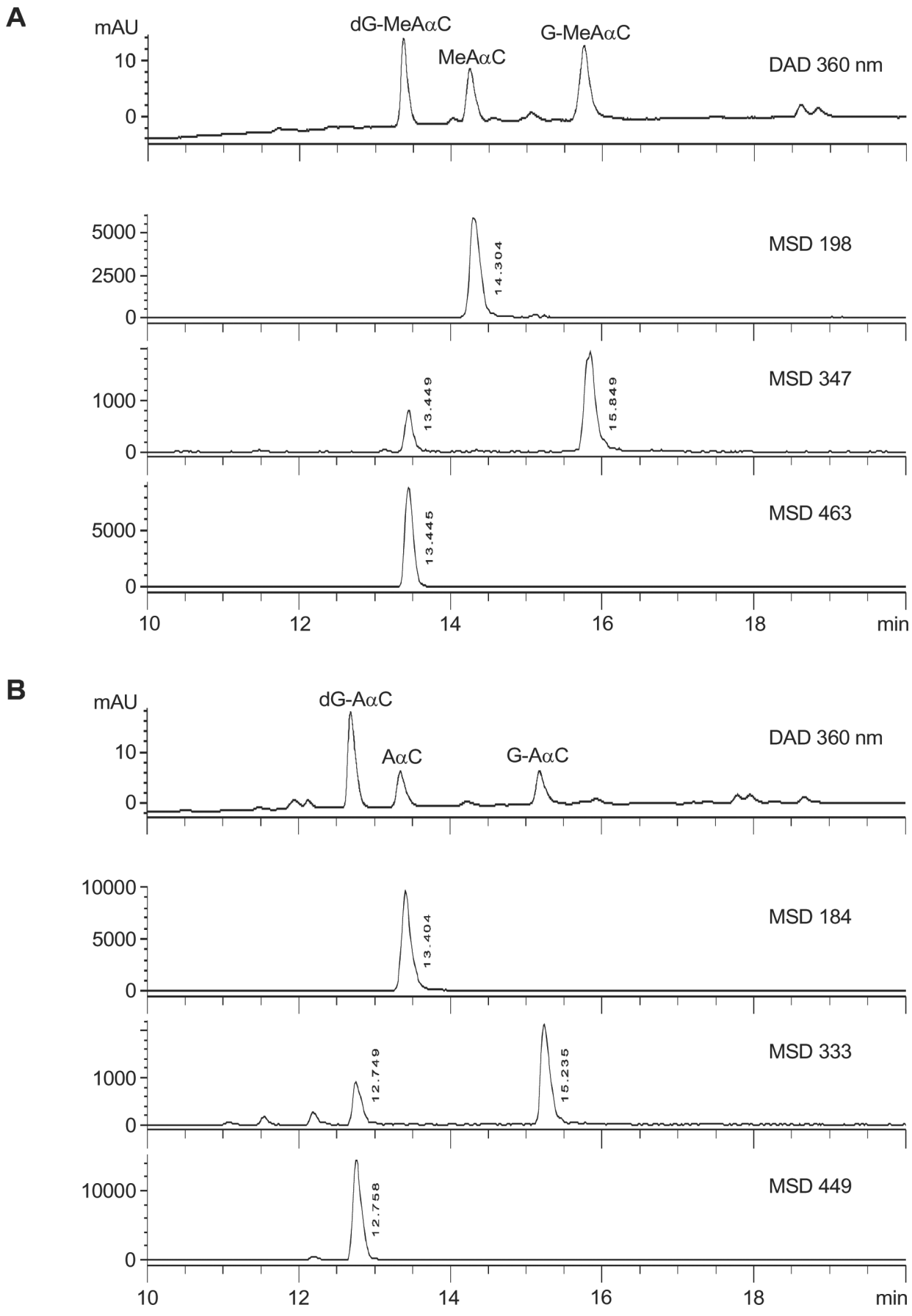

Fig. 1. UV chromatogram of the crude reaction mixtures of 2′-deoxyguanosine and (**A**) acetylated N^2-OH-MeAαC and (**B**) acetylated N^2-OH-AαC. The extracted ion chromatograms show MeAαC (*m*/*z* 198), G-MeAαC (*m*/*z* 347), dG-MeAαC (*m*/*z* 463), AαC (*m*/*z* 184), G-AαC (*m*/*z* 333) and dG-AαC (*m*/*z* 449).

15.8 min. The first eluting compound was identified as dG-MeAαC. Mass spectral analysis of this compound showed a molecular ion $[M + H]^+$ at *m/z* 463 with a daughter ion $[M + H]^+$ at *m/z* 347. Loss of the 116 Da component indicates cleavage of the deoxyribose ring from dG-MeAαC. Mass spectral analysis of the third eluting compound showed a molecular ion $[M + H]^+$ at *m/z* 347 (no daughter ions), which was consistent with an adduct between MeAαC and guanine formed by loss of deoxyribose from the dG-MeAαC adduct. The chromatographic profile of dG-AαC (Figure 1B) showed a similar pattern; a major compound eluting before AαC at 12.7 min and a second major compound eluting after AαC at 15.2 min. The first eluting compound was identified as dG-AαC. Mass spectral analysis of this compound showed a molecular ion $[M + H]^+$ at *m/z* 449 with a daughter ion $[M + H]^+$ at *m/z* 333, indicating cleavage of the deoxyribose ring from dG-AαC. Mass spectral analysis of the third eluting compound showed a molecular ion $[M + H]^+$ at *m/z* 333 (no daughter ions), which was consistent with an adduct between AαC and guanine formed by loss of deoxyribose from the dG-AαC adduct.

To confirm that the third eluting compounds were guanin-8-yl-adducts, G-MeAαC and G-AαC were synthesized for comparison by a reaction between acetylated guanine *N*3-oxide and the parent compounds (25,26). HPLC analyses of G-MeAαC and G-AαC synthesized from guanine *N*3-oxide showed HPLC retention times, UV spectra (Figure 2) and mass spectra identical to the two third eluting peaks from the reaction between dG and N^2-OH-MeAαC and N^2-OH-AαC, respectively, corroborating the identity of those adducts. Figure 2 shows that the guanine adducts had different UV spectra than the deoxyguanosine adducts. The structures of G-MeAαC and G-AαC have previously been characterized by HPLC-MS and NMR analyses (25).

The deoxyguanosine adducts appeared to be rather unstable and easily lost the deoxyribose moiety, particularly at low pH. The pH values in the deoxyguanosine adduct mixtures after final reaction were about 3–4. Elevation of the pH in the reaction product mixtures to >7 dramatically improved the stability of the deoxyguanosine adducts. Figure 3 shows the decomposition of dG-AαC over time at various pH values. The rapid decomposition of the deoxyguanosine adducts at pH values <7 could be reduced by storing the deoxyguanosine adducts at $-20°C$. Figure 4 show the UV chromatograms of dG-MeAαC stored at 37°C for 20 h at different pH values. At pH 5.4 nearly all dG-MeAαC was decomposed to G-MeAαC, while only a small amount of the dG-MeAαC was decomposed at pH 7.4. The decomposition of dG-AαC was complete at pH 5.3, while the compound was well conserved at pH 7.3. The decomposition of guanine adducts was in accordance with previous studies, where acid hydrolysis of modified DNA resulted in similar decomposition (25).

Formation of N^2-(3′-phospho-2′-deoxyguanosin-8-yl) adducts and modification of calf thymus DNA

Addition of N^2-OH-MeAαC or N^2-OH-AαC to solutions of calf thymus DNA resulted in only a low degree of modification of the DNA. High yields of adducts were obtained by acetylation of the N^2-OH derivatives prior to addition to deoxyguanosine. Addition of N^2-acetoxy derivatives to calf thymus DNA resulted in only a low degree of modification of the DNA. Due to the high viscosity of the reaction mixture, the N^2-acetoxy derivates reacted before being properly mixed with the DNA. However, addition of the N^2-OH derivatives to the DNA solution followed by addition of acetic anhydride resulted in a much higher degree of modification of the calf thymus DNA. Room temperature incubation of N^2-OH-MeAαC and

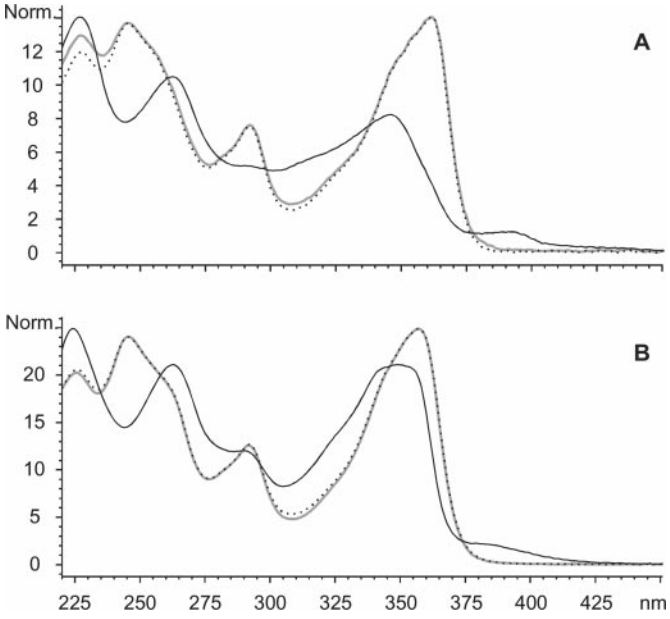

Fig. 2. Comparison of UV spectra of MeAαC compounds (**A**) and AαC compounds (**B**). Black lines show dG adducts and black dotted lines show G adducts, all from synthesis of dG adducts. Grey lines show G adducts synthesized with guanine-N^3-oxide.

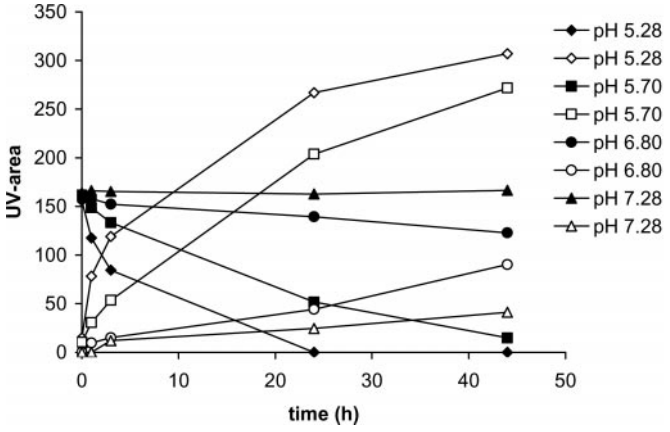

Fig. 3. Decomposition of dG-AαC over time stored at various pH values. Filled symbols show dG adducts and open symbols show G adducts.

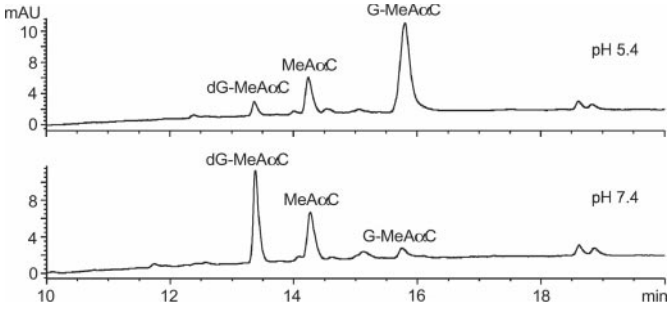

Fig. 4. UV chromatograms of dG-MeAαC stored at 37°C for 20 h at pH 5.4 and pH 7.4.

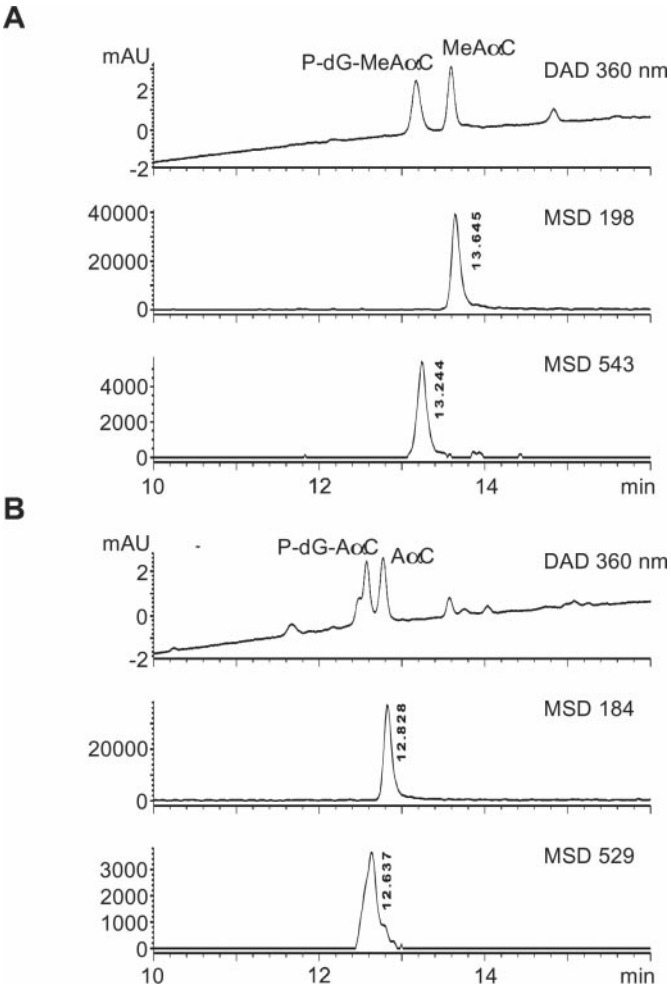

Fig. 5. UV chromatogram of the crude reaction mixture of 3′-phospho-2′-deoxyguanosine and acetylated N^2-OH-MeAαC (**A**) and acetylated N^2-OH-AαC (**B**). The extracted ion chromatograms show MeAαC (*m/z* 198), P-dG-MeAαC (*m/z* 543), AαC (*m/z* 184) and P-dG-AαC (*m/z* 529).

N^2-OH-AαC with 3′-phospho-2′-deoxyguanosine at neutral pH followed by addition of acetic anhydride also resulted in P-dG-MeAαC and P-dG-AαC adducts. Figure 5 show the HPLC profiles of P-dG-MeAαC and P-dG-AαC. In addition to MeAαC, the chromatogram (Figure 5A) shows one major compound eluting at 13.2 min. Mass spectral analysis of this compound showed a molecular ion $[M + H]^+$ at *m/z* 543, consistent with a P-dG-MeAαC adduct. The chromatogram of P-dG-AαC (Figure 5B) showed a similar pattern, with a major compound eluting before AαC at 12.6 min, and mass spectral analysis of this compound showed a molecular ion $[M + H]^+$ at *m/z* 529, consistent with a P-dG-AαC adduct. The 345 Da increase in molecular weight of the adducts of MeAαC and AαC is in accord with addition of a 3′-phospho-2′-deoxyguanosine group. The 3′-phospho-2′-deoxyguanosine adducts were also unstable in different buffer solutions with pH < 7.2. The half life ($t_{½}$) of P-dG-MeAαC at pH 5.4 at 37°C was ~6 h and the $t_{½}$ of P-dG-AαC under similar conditions was ~5 h (data not shown). The pH values in the final reaction mixtures were ~5–6, therefore, it was necessary to increase the pH value in the reaction mixtures before storing.

After precipitation, modified calf thymus DNA was digested by nuclease P1 and alkaline phosphatase. HPLC analysis of the digests monitored at 260 nm and mass spectral analyses showed four major compounds identified as the four unmodified deoxynucleosides, indicating successful enzymatic hydrolysis of the modified DNA. HPLC analysis of the digests monitored at 360 nm followed by mass spectral analysis showed major compounds identified as dG-MeAαC or dG-AαC. These compounds had elution times, UV spectra and masses identical to synthetic dG-MeAαC and dG-AαC, respectively. Figure 6 shows UV chromatograms of different purification fractions of digested calf thymus DNA modified with AαC.

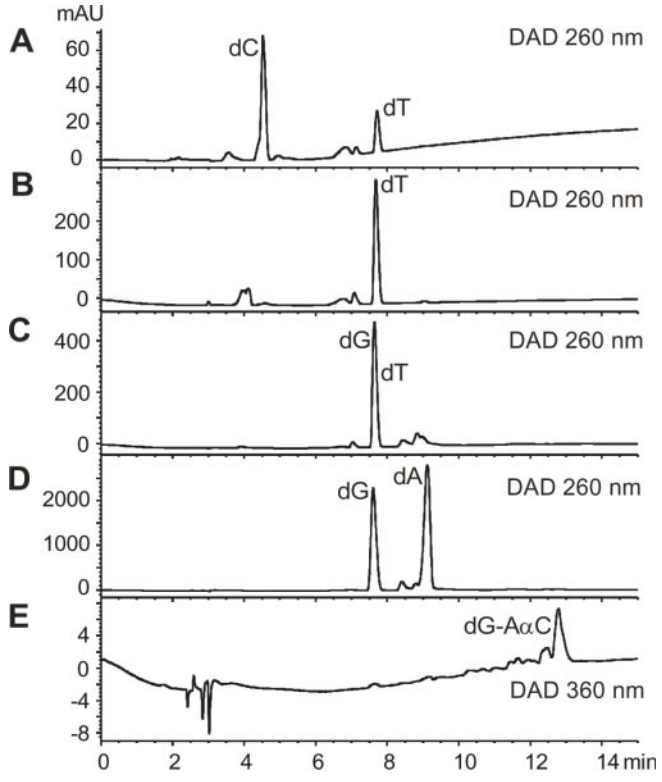

Fig. 6. UV chromatograms of digested calf thymus DNA modified with AαC purified on a Isolute colum. (**A**) Flow-through fraction; (**B**) wash fraction; (**C**) eluate 1; (**D** and **E**) eluate 2 monitored at 260 and 360 nm.

^{32}P-postlabelling of 3′-phospho-2′-deoxyguanosine adducts and modified calf thymus DNA

^{32}P-postlabelling analysis of the synthesized 3′-phospho-2′-deoxyguanosine adduct standards resulted in one major adduct spot of dG-MeAαC and one major adduct spot of dG-AαC (Figure 7A and C). The mean RAL of duplicate analyses were 0.18 and 0.40 per 10^6 2′-deoxyguanosine for P-dG-MeAαC and P-dG-AαC, respectively (Table I). Three adducts were detected by ^{32}P-postlabelling analysis of digested calf thymus DNA modified with MeAαC (Figure 7B). The major adduct co-migrated with synthetic dG-MeAαC (Figure 7A). The mean RAL of duplicate analyses was 0.76 per 10^6 nt for the major adduct (identified as dG-MeAαC) and the total adduct level was 1.4 per 10^6 nt (Table I). ^{32}P-postlabelling analysis of digested calf thymus DNA modified with AαC resulted in two adducts (Figure 7D), with the major adduct co-migrating with synthetic dG-AαC (Figure 7C). Both the RAL and the total adduct level of calf thymus DNA modified with AαC were ~5-fold higher than the adduct level in calf thymus modified with MeAαC. The mean RAL of duplicate analyses was 6.0 per

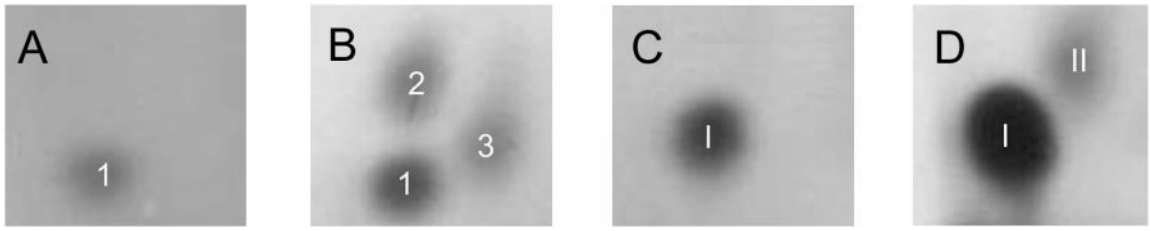

Fig. 7. Autoradiograms of ^{32}P-postlabelling/TLC analyses of the standard P-dG-MeAαC (**A**), calf thymus DNA modified with MeAαC (**B**), the standard P-dG-AαC (**C**) and calf thymus DNA modified with AαC (**D**). The major adduct spots 1 and I are dG adducts of MeAαC and AαC, respectively.

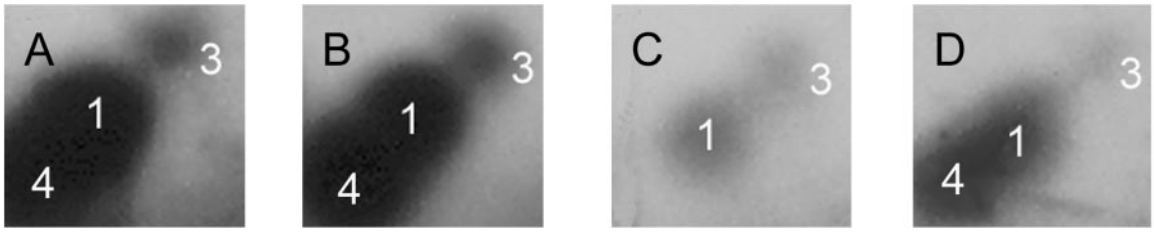

Fig. 8. Autoradiograms of ^{32}P-postlabelling/TLC analyses of DNA isolated from liver (**A**), colon (**B**), kidney (**C**) and heart (**D**) from a rat dosed with MeAαC. The major adduct spot 1 was identified as dG-MeAαC. Autoradiography was overnight at −80°C.

Table I. Relative adduct level (RAL × 10^6) in the dG adduct standard and modified calf thymus DNA preparations analysed by ^{32}P-postlabelling[a]

	dG adduct standard	Calf thymus DNA
dG-MeAαC	0.1756	0.7611
Adduct 2		0.3260
Adduct 3		0.2997
Total adduct level	0.1756	1.3868
dG-AαC	0.4001	5.9929
Adduct 2		0.8150
Total adduct level	0.4001	6.8080

[a]Values are the means of duplicates.

10^6 nt for the major adduct (identified as dG-AαC) and the total adduct level was 6.8 per 10^6 nt (Table I). The identity of the major adducts formed by incubation of the N^2-OH derivatives with 3′-phospho-2′-deoxyguanosine and calf thymus DNA was confirmed by HPLC analysis of ^{32}P-labelled adducts (25). The minor adduct spots of the modified calf thymus DNA were proposed to be other unidentified adducts or undigested oligomers, probably caused by incomplete hydrolysis of the DNA (36).

^{32}P-postlabelling of MeAαC–DNA formed in vivo

Analysis of DNA isolated from liver, colon, kidney and heart of the three rats dosed with [^{3}H]MeAαC by ^{32}P-postlabelling resulted in three detectable adduct spots from each tissue sample (Figure 8). One of the major adducts in all samples co-migrated with synthetic P-dG-MeAαC and the major adduct in calf thymus DNA modified with MeAαC, therefore this adduct was identified as dG-MeAαC. Two other adducts present in modified calf thymus DNA were also detected, except in the kidney, where only one minor adduct was detected. The highest relative adduct level ~50.4 adducts/10^9 nt was found in the liver. The lowest RAL was detected in the kidney, ~12-fold lower than in the liver at ~4.19 adducts/10^9 nt (Table II). In digested DNA from colon and heart tissues the RALs were found to be 4-fold lower than the RALs in the liver tissue, at 12.4 and 14.0 adducts/10^9 nt, respectively.

Table II. Total MeAαC–DNA adduct levels in tissue from rats dosed with 8.6 mg/kg MeAαC

Tissue	RAL × 10^9 (mean ± SD)[a]
Liver	50.40 ± 13.67
Colon	12.42 ± 4.40
Kidney	4.19 ± 1.25
Heart	13.98 ± 8.82

[a]Values are the means ± SD of three independent preparations in each tissue from three rats.

Discussion

Following activation with acetic anhydride, the proximate mutagenic N^2-hydroxylated metabolites of the food mutagens MeAαC and AαC (N^2-OH-MeAαC and N^2-OH-AαC) bind covalently to 2′-deoxyguanosine, but not to the other deoxynucleosides. This is in accordance with other studies of heterocyclic amine adduct formation, including AαC, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQ), 2-amino-4,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethyl-imidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), 2-amino-6-methylpyrido[1,2-a:3′,2′-*d*]imidazole, 3-amino-1-methyl-5*H*-pyrido[3,4-*b*]indole and PhIP, which all form major adducts where the amine is bound to the C8 carbon atom of this base (14,27,37–39).

Acetylation of the N^2-OH compounds with acetic anhydride resulted in formation of the putative N^2-acetoxy derivatives. The instability of these compounds prevented analysis. However, following reaction with deoxyguanosine resulting in formation of adducts, identified as dG-MeAαC and dG-AαC (Figure 9 shows the reaction scheme), the highest yields were obtained when acetylation was performed at low pH at −20°C. The N^2-acetoxy derivatives were added in small portions to a rapidly stirred, pH neutral solution of 2′-deoxyguanoside at room temperature. Previously it was shown that the highest yields of N^2-acetoxy-PhIP and N^2-acetoxy-4,8-DiMeIQx were formed at 0 and −50°C, respectively, followed by dG-PhIP formation at 37°C and formation of dG-4,8-DiMeIQx at 0°C,

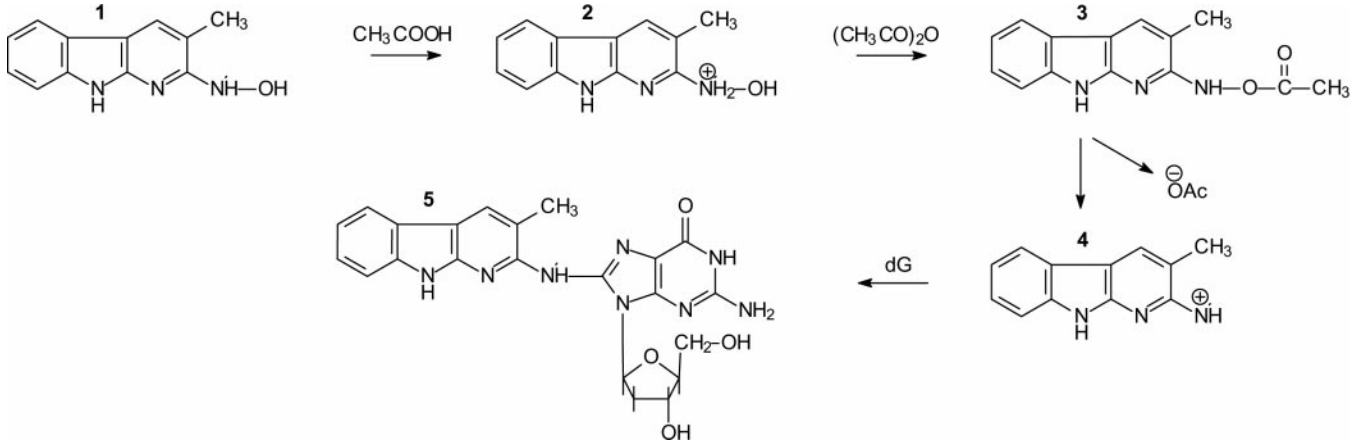

Fig. 9. Reaction scheme: MeAαC (**1**), protonated MeAαC (**2**), N^2-acetoxy derivative (**3**), nitrenium ion of MeAαC (**4**) and N^2-(2′-deoxyguanosine-8-yl)-MeAαC (**5**).

resulting in the highest yields of these compounds (27,37). These differences indicate that the reaction temperature for both formation of the N^2-acetoxy derivatives and formation of deoxyguanosine adducts is an extremely important parameter in the syntheses of deoxyguanosine adducts of heterocyclic amines.

The deoxyguanosine adducts were characterized as dG-MeAαC and dG-AαC, respectively, and were identical to previously characterized deoxyguanosine C8 adducts of MeAαC and AαC formed by reaction of the parent amine with acetylated guanine *N*3-oxide (25,26). Activation of *N*-OH-IQ and *N*-OH-MeIQx by acetic anhydride has been shown to result in formation of minor adducts in addition to the major deoxyguanosine C8 adducts. In these minor adducts the C5 carbon atom of the heterocyclic amine is attached to the N2 atom of guanine (40). In the present investigation, following a similar activation procedure, the presence of minor adducts in addition to the deoxyguanosine C8 adducts was not detected.

Unfortunately, the method used for high yield synthesis of deoxyguanosine adduct did not result in detectable modification of calf thymus DNA. However, addition of acetic anhydride to a solution of calf thymus DNA containing small amounts of N^2-OH-MeAαC or N^2-OH-AαC resulted in low, but detectable, amounts of deoxyguanosine adducts.

In contrast to deoxyguanosine adducts of other heterocyclic amines, such as dG-PhIP, dG-MeIQx and dG-4,8-DiMeIQx, both the synthetic deoxyguanosine adducts and the synthetic 3′-phospho-2′-deoxyguanosine adducts of MeAαC and AαC were very unstable at pH values <7. The pK_a of AαC is 4.40, compared with 5.56 for PhIP and 5.95 for MeIQx (41). The lower electron donating ability of the amino group of AαC compared with PhIP and MeIQx may result in a lower electron density in the neighbouring glycosidic bond, making it more labile.

The $t_{½}$ values of P-dG-MeAαC and P-dG-AαC at pH 5.4 at 37°C were ~6 and 5 h, respectively. ^{32}P-postlabelling analysis of DNA from tissue samples involves several purification/digestion steps carried out at low pH for prolonged times. The apparent instability of the MeAαC and AαC adducts at low pH may therefore result in false low values of adducts as measured by ^{32}P-postlabelling.

In the ^{32}P-postlabelling analysis of the synthetic 3′-phospho-2′-deoxyguanosine adducts only one adduct spot of dG-MeAαC and one adduct spot of dG-AαC were detected, which was in accord with the HPLC-MS analysis of these adducts. The 3′-phospho-2′-deoxyguanosine adducts co-migrated with the major adduct spot of modified calf thymus DNA and with the major adduct spot from DNA of rats dosed with MeAαC, indicating that the major adducts formed *in vitro* and *in vivo* are dG-MeAαC and dG-AαC, respectively.

In addition to the major adduct spot, identified as dG-MeAαC, detected by ^{32}P-postlabelling analysis of MeAαC-modified calf thymus DNA, two minor adducts proposed to be oligomers or other unidentified adducts were observed, accounting for ~45% of the total adduct level. Such minor adduct spots are often detected in ^{32}P-postlabelling analyses and are probably caused by incomplete hydrolysis of the DNA (36). ^{32}P-postlabelling analysis of AαC-modified calf thymus DNA showed one minor adduct accounting for ~12% of the total adduct level.

The DNA from tissues of the three rats dosed with MeAαC showed three adducts by ^{32}P-postlabelling analyses. One of these adducts was identified as dG-MeAαC. The largest total adduct level was found in the DNA from the liver of the rats, ~50.40 adducts/10^9 nt. A 4-fold lower level of total adducts were found in the DNA from colon and heart and a 12-fold lower level of total adduct was found in the kidney. These levels were much lower than the total adduct level found in tissues of F344 female rats fed 800 p.p.m. MeAαC acetate in the diet. ^{32}P-postlabelling analyses showed one major adduct and up to 16 minor adducts. The total adduct level was 1 per 10^5 nt in the liver and 2- to 3-fold lower in other organs, such as kidney, lung, spleen and salivary glands (24). However, a direct comparison is difficult, due to the difference in MeAαC administration, dose and sampling time. In this study DNA was isolated from tissues from rats receiving a continuous dose of MeAαC in the feed. In the present study DNA was isolated from tissues 3 days after a single oral dose of MeAαC. Therefore, a lower adduct level in the organs was expected, as shown in a previous study, where adduct levels in the liver decreased by a factor of 14 from 2 to 72 h after dosing with PhIP (42). In the present study the highest adduct level was found in the liver. This was in accord with other *in vivo* studies of adduct levels of MeAαC, AαC, MeIQ and IQ in rodents (14,24,43).

In conclusion, activation of the N^2-OH derivatives of MeAαC and AαC results in formation of deoxyguanosine adducts identified as dG-MeAαC and dG-AαC, respectively. These adducts were also detected as the major adducts in calf

thymus DNA modified *in vitro*. Finally, dG-MeAαC was the major adduct in DNA from rats treated with MeAαC.

Acknowledgements

The authors thank Helle E.Gluver and Klaus Söhren for skilled technical assistance. This study was carried out with financial support from the commission of the European Communities, specifically the RTD programme 'Quality of life and management of living resources' QLK1-CT99-01197, Heterocyclic amines in cooked foods—role in human health. It does not necessarily reflect its views and in no way anticipates the commission's future policy in this area.

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Received December 10, 2003; revised March 19, 2004; accepted March 22, 2004