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In vitro Metabolism of Two Heterocyclic Amines, 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) in Human and Rat Hepatic Microsomes

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Abstract: 2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC) are two mutagenic and carcinogenic heterocyclic amines formed during ordinary cooking. In this study, we have investigated the *in vitro* metabolism of tritium-labelled AαC and MeAαC in hepatic microsomes from human pools, rats induced with polychlorinated biphenyl (PCB) (Aroclor 1254) and control rats. The microsomes were incubated with AαC and MeAαC and the detoxified and activated metabolites of AαC and MeAαC were separated and characterised by HPLC-MS. AαC is metabolised to two major and three minor detoxified metabolites, while MeAαC is metabolised to three major and one minor detoxified metabolites. Some AαC and MeAαC are activated by oxidation to the reactive metabolites N^2-OH-AαC and N^2-OH-MeAαC, respectively. These reactive N^2-OH-metabolites react partially in the incubation system with formation of protein adducts, dimers and the parent compound by reduction of the N^2-OH-metabolites. The distribution between the detoxified and activated metabolites in the different types of hepatic microsomes showed same pattern for both AαC and MeAαC. In PCB-induced rat microsomes, the major part of the metabolites are detoxified, only a little amount is activated. In control rat microsomes there is a fifty-fifty distribution between detoxification and activation, while the major part of the metabolites from the human microsomes are activated and reacts to form dimers and protein adducts. These data show that, in human hepatic microsomes compared to rat hepatic microsomes, a major part of AαC and MeAαC are metabolically activated to the reactive N^2-OH-AαC and N^2-OH-MeAαC.

2-Amino-9*H*-pyrido[2,3-*b*]indole (AαC) and its methyl homologue, 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC), are two food-borne mutagenic and carcinogenic heterocyclic amines. AαC and MeAαC are often referred to as α-carbolines (fig. 1). AαC and MeAαC are formed as pyrolysis products of tryptophan-rich compounds and are also found in cooked food such as meat, chicken and fish (Gross & Gruter 1992; Holder *et al.* 1997; Skog *et al.* 1998; Solyakov *et al.* 1999) and in cigarette smoke condensates (Yoshida & Matsumoto 1980; Matsumoto *et al.* 1981; Manabe *et al.* 1990; Wakabayashi *et al.* 1995). MeAαC is also found in wine (Richling *et al.* 1997). AαC and MeAαC are mutagenic in bacterial test systems (Nagao *et al.* 1983; Holme *et al.* 1989; Wild *et al.* 1991). Dietary administration of AαC and MeAαC to mice and rats induced liver tumours (Ohgaki *et al.* 1984; Hasegawa 1992). AαC and MeAαC have, like other heterocyclic amines, two metabolic pathways, detoxification or activation. The first step in the metabolism of the heterocyclic amines is a phase I hydroxylation catalysed by cytochrome P450 enzymes. Detoxified compounds are often ring-hydroxylated followed by phase II conjugation. Activated compounds are hydroxylated in their characteristic exocyclic amino group, usually followed by O-esterification catalysed by e.g. acetyltransferase or sulfotransferase (Eisenbrand & Tang 1993). Especially enzymes from the CYP1A-family are involved in the phase I metabolism of AαC and MeAαC to their corresponding N^2-OH-derivates (Niwa *et al.* 1982; Sugimura 1985). Activated heterocyclic amines are able to form adducts with macromolecules such as protein and DNA. It is shown by ^{32}P-post-labeling analysis that MeAαC and AαC form one major DNA adduct (N^2-deoxyguanin-8-yl-(Me)AαC) in primary hepatocytes from rats (Pfau *et al.* 1996 & 1997).

There are only few reports of the metabolism of AαC and MeAαC compared with other heterocyclic amines. Recently some of the major metabolites of AαC in human and rodent hepatic microsomes were characterized by Raza *et al.* (1996) and King *et al.* (2000). Previously, the metabolism of MeAαC in hepatic microsomes from PCB-induced rat was studied (Frandsen *et al.* 1998), but not the metabolism of MeAαC in human microsomes.

The aim of this study was to investigate the *in vitro* metabolism of AαC and MeAαC in hepatic microsomes from rat and man. In the present study we have investigated the *in vitro* metabolism of AαC and MeAαC in hepatic micro-

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somes from polychlorinated biphenyl (PCB)-induced rats, control rats and two different human pools and compared the distribution between activation and detoxification of the metabolites. N^2-OH-derivaties of both AαC and MeAαC were synthesized for comparison with metabolites of the two heterocyclic amines.

Materials and Methods

Chemicals. AαC, [^{3}H]-AαC and MeAαC were obtained from Toronto Research Chemicals (Toronto, Canada). Tritiation of MeAαC is described previously (Frandsen *et al.* 1998). Pooled human liver microsomes (from 15 donors) were obtained from *in vitro* Technologies (Baltimore, MD, USA) and pooled human liver microsomes (from 11 donors) was obtained from Gentest (Woburn, MA, USA). Polychlorinated biphenyl (PCB: Aroclor 1254) was obtained from Monsanto Industrial Chemical Co. (St. Louis, MO, USA). Isocitricdehydrogenase (from pork heart), nicotinamide adeninedinucleotide-phosphate and 3-[N-morpholino] propanesulfonic acid hemi sodium salt (MOPS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile and formic acid (distillated before use) were obtained from Romil (Cambridge, UK). Chromabond columns (C18ec and 2OH) were obtained from Macherey-Nagel (Duren, Germany). Soluene-350 and Hionic-Flour were obtained from Packhard (Meriden, CT, USA). All other chemicals were of analytical purity and obtained from Riedel-de Haën (Seelze, Germany), Merck (Darmstadt, Germany), Fisher Scientific (Loughborough, UK) and Rathburn (Walkerburn, Scotland, UK).

Synthesis of 2-nitro-3-methyl-9H-pyrido[2,3-b]indole (NO_2-MeAαC). A previously described method (Frandsen *et al.* 1998) was modified in the following way: A sample of 3.4 mg MeAαC was dissolved in 1 ml acetic acid and heated to 90°. One hundred µl of hydrogen peroxide (30%) was added drop-wise under stirring. Heating and stirring were continued for 30 min. The reaction mixture was diluted with 15 ml water and applied in small portions onto an activated Chromabond C18ec column. The column was washed with 5 ml water and eluted with 2 ml methanol. The eluate was placed in a 50° water bath and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 2 ml of ethyl acetate and added 6 ml of heptane. The dissolved part of the residue was applied onto a Chromabond 2OH column equilibrated with heptane. The eluate was collected and the column was eluted with 2 ml ethyl acetate/heptane (1:1). The combined eluate was evaporated as described above, redissolved in 1 ml dimethyl sulfoxide (DMSO) and stored at 4°. The product was diluted in 1% formic acid in 10% acetonitrile and analysed by high performance liquid chromatography-mass spectrometry (HPLC-MS). The yield of NO_2-MeAαC was 1.6 mg (41%).

Synthesis of 2-hydroxyamino-3-methyl-9H-pyrido[2,3-b]indole (N^2-OH-MeAαC). A previously described method (Agus *et al.* 2000) was modified in the following way: All solvents were purged with argon before use and all solutions were stored under argon. Two hundred µl water was added to 1 ml 0.2 M ascorbic acid in DMSO. The solution was stirred at room temperature for 5 min. Forty µl 0.3 M copper sulphate was added drop wise under stirring. After 5 min. with stirring, 0.5 ml of 0.94 mg/ml NO_2-MeAαC in DMSO was added drop-wise (30 sec.) under stirring. After 45 sec. the reaction was stopped by addition of 2 ml 0.1 M EDTA followed by 8 ml ice-cold water under stirring. The reaction mixture was kept on ice until purification. The reaction mixture was under argon applied in small portions on an activated Chromabond C18ec column. The column was washed with 4 ml ice-cold water, 2 ml ice-cold 10% methanol and 2 ml ice-cold water. N^2-OH-MeAαC was eluted with 1.2 ml ice-cold DMF and stored at –20°. The synthesis product was diluted in 1% formic acid in 10% acetonitrile and analysed by HPLC-MS. The yield of N^2-OH-MeAαC was 192 µg (44%).

Synthesis of 2-nitro-9H-pyrido[2,3-b]indole (NO_2-AαC). NO_2-AαC was synthesised by the same method as the synthesis of N^2-MeAαC. 3.6 mg AαC was used and the yield of NO_2-AαC was 1.6 mg (38%).

Synthesis of 2-hydroxyamino-9H-pyrido[2,3-b]indole (N^2-OH-AαC). N^2-OH-AαC was synthesised by the same method as the synthesis of N^2-OH-MeAαC. 0.4 mg NO_2-AαC was used and the yield of N^2-OH-AαC was 110 µg (59%).

Synthesis of MeAαC- and AαC-dimers. N^2-OH-MeAαC dissolved in dimethyl formamid (DMF) was diluted to 50 µg/ml with argon-purged water. To the solution was added 5 µl acetic anhydride under a stream of argon. The reaction mixture was kept under argon and stirred at room temperature for 3 hr. MeAαC treated in the same way was used as reference. The syntheses products were diluted in argon-purged 1% formic acid in 10% acetonitrile and analysed by HPLC-MS. The same procedure was used to create AαC-dimers.

Preparation of rat hepatic microsomes. Adult male Wistar rats (age 7–8 weeks, weight ~ 200 g) were delivered from Møllegaard Breeding Center (Lille Skensved, Denmark). Microsomes from PCB-induced rats and control rats were prepared as previously described (Frandsen *et al.* 1994). Four rats were used to prepare a pool of PCB-induced hepatic rat microsomes. PCB (Aroclor 1254, 500 mg/kg, dissolved in corn oil) was injected intraperitoneally 5 days before sacrifice. Two rats were used to prepare a pool of control hepatic rat microsomes.

In vitro metabolism. The microsomal incubations were performed as previously described (Frandsen *et al.* 1998). Five µg test substance (MeAαC, [^{3}H]-MeAαC, AαC and [^{3}H]-AαC dissolved in DMF) per ml incubation mixture was added after preincubation for 5 min. The reactions were terminated after 30 min. for the MeAαC incubations and after 45 min. for the AαC incubations by addition of one volume of ice-cold argon-purged ethanol. After centrifugation the supernatants were isolated and analysed by HPLC-MS.

Analytical. High performance liquid chromatography analyses were performed on a Agilent Technologies model 1100 liquid chromatograph equipped with a photodiode array detector (Agilent Technologies, Wallbronn,Germany). The products were separated on a Zorbax SB-C3, 5 µm, 150×3 mm column from Agilent Technologies. The flow rate was 0.4 ml/min. and the oven temperature was 40°. Solvent were A: 1% formic acid and B: acetonitrile. Solvent programming was: 0–2 min., 2% B; 10 min., 25% B; 20 min., 80% B; 22 min., 2% B.

Positive ion electrospray mass spectra were obtained with an Agilent Technologies MSD 1100 mass spectrometer equipped with an electro-spray interface (Agilent Technologies, Wallbronn, Germany). The following interphase settings were used: nebulizer pressure 60 psi; drying gas(nitrogen) 10 l/min., 350°; capillary voltage 4000 V; fragmentor voltage 70 V.

When the incubations were conducted with tritiated materials the supernatants were analysed by HPLC and 1 min. fractions were collected (the same HPLC-conditions were used to analyse both tritiated and not-tritiated materials). Four ml Hionic-Flour were added to the collected fractions followed by liquid scintillation counting. The precipitates from the incubations with tritiated materials were washed twice with acetone and dissolved in Soluene-350. One hundred µl of the solutions were added 4 ml of Hionic-Flour and analysed by liquid scintillation counting. Liquid scintillation counting was performed on a Tri Carb 3100TR using a Hion-

ic-Flour scintillation cocktail and with external standardization (Packhard, Meriden, CT, USA).

All incubations were repeated three times with similar results.

Results

Metabolism of MeAαC and AαC. Several metabolites of MeAαC and AαC were detected in microsomes from PCB-induced rats, control rats and humans after incubation with MeAαC and AαC. Fig. 2 shows the HPLC profiles of MeAαC and AαC and their metabolites from the microsomal incubations. Two different pools of human microsomes were used, but there were no particular difference between the metabolism of MeAαC and AαC in the two human hepatic microsomal pools.

In PCB-induced hepatic rat microsomes MeAαC is metabolised to three major metabolites (labelled 1–3) and one minor metabolite (labelled with arrow) eluting before the parent compound, MeAαC (fig. 2A). The mass spectra of all the early eluting metabolites showed molecular ions $[M+H]^+$ at *m/z* 214, indicating that they are hydroxylated metabolites. An earlier identification of the three major metabolites by electro spray mass spectrometry and ^{1}H-NMR has shown, that metabolite 1, 2 and 3 can be characterized as 6-OH-MeAαC, 3-CH_2OH-AαC and 7-OH-MeAαC, respectively (Frandsen *et al.* 1998). UV spectra of the minor metabolite were nearly identical with the UV spectra of 6-OH-MeAαC and 7-OH-MeAαC (data not shown), therefore it is proposed that the minor metabolite could be another ring-hydroxylated metabolite. The tree early eluting major metabolites are all characterized as detoxified compounds (Frandsen *et al.* 1998). Small amounts of two other metabolites (labelled 4 and 5) are eluting after MeAαC. The mass spectra of the late eluting metabolites showed molecular ions $[M+H]^+$ at *m/z* 393, both with daughter ions $[M+H]^+$ at *m/z* 197. This high molecular weight indicates that dimers of a reactive metabolite are formed. The presence of a reactive metabolite was not detected during HPLC analysis, but its presence is indicated by binding of 8% of the radioactivity to the protein pellet.

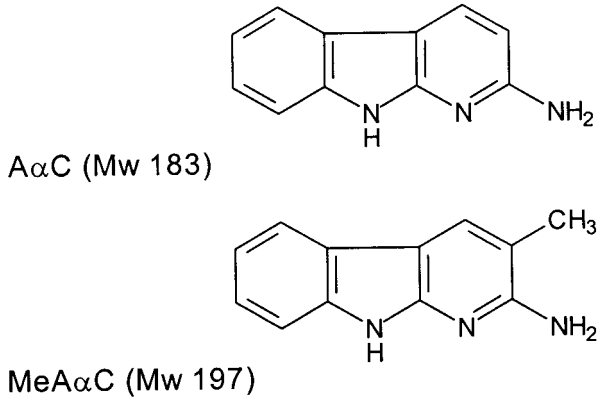

Fig. 1. Structure and molecular weights of 2-amino-9*H*-pyrido[2,3-*b*]indole (AαC) and 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeAαC).

Similar metabolite patterns of MeAαC are found in incubation with control rat and human microsomes (fig. 2B and 2C). However, the amounts of the late eluating metabolites 4 and 5 in control rat and human microsomes are considerable larger than the amount of metabolites 4 and 5 in PCB-induced rat microsomes.

Fig. 2D, E and F shows the metabolism of AαC in hepatic microsomes from PCB-induced rats, control rats and man. Also here a similar metabolite pattern in the three different types of microsomes is obtained. In PCB-induced rat and control rat microsomes AαC are metabolised to three major metabolites (labelled 1–3) and two minor metabolites (labelled with arrows) eluting before the parent compound, AαC. In human hepatic microsomes it is primarily metabolite 1 and 3 that are dominating, but also metabolite 2 is noticeable. The mass spectra of all the early eluting metabolites showed molecular ions $[M+H]^+$ at *m/z* 200, indicating that they are hydroxylated metabolites. Raza *et al.* (1996) have characterized the major metabolites of AαC in human microsome incubation as 3-OH-AαC and 6-OH-AαC. UV spectra of metabolite 1 and 3 were identical with the published UV spectra of 6-OH-AαC and 3-OH-AαC, respectively (data not shown). In the three chromatograms (fig. 2D, E and F) two other metabolites (labelled 4 and 5) are eluting after AαC. The mass spectra of the late eluting metabolites showed molecular ions $[M+H]^+$ at *m/z* 365, both with daughter ions $[M+H]^+$ at *m/z* 183, indicating that dimers of a reactive metabolites is formed. The last metabolite (labelled 6), eluting right before the parent compound, AαC, was found in the human microsomal incubations (fig. 2F). A small amount of metabolite 6 was also detectable in the preparation with PCB-induced rat microsomes by analysis of the MS data using extracted ion chromatogram (data not shown). Metabolite 6 has a molecular ion $[M+H]^+$ at *m/z* 200 with a daughter ion $[M+H]^+$ at *m/z* 183. This metabolite was much more unstable, when stored in air at room temperature, than the other metabolites (labelled 1–5), which could indicate that this metabolite was the reactive N^2-OH-AαC.

Synthesis of N-OH-MeAαC and N-OH-AαC. In order to evaluate the possible presence of unstable N^2-hydroxylated metabolites, N^2-OH-MeAαC and N^2-OH-AαC were synthesised by two-step procedures. MeAαC and AαC were oxidized to NO_2-derivates with H_2O_2 in acetic acid. In an attempt to increase the yield of NO_2-MeAαC, we have tried to use different reaction conditions. However, oxidation of MeAαC with dimethyldioxirane, a method developed to synthesize NO_2-AαC (King *et al.* 2000), or oxidation with H_2O_2 of MeAαC diluted in triflour acetic acid, trichlor acetic acid, formic acid, diluted sulphuric acid and diluted phosphoric acid did not result in higher yields.

In the second step the NO_2-compounds were reduced to N^2-OH-MeAαC and N^2-OH-AαC using copper sulphate in ascorbic acid. These methods were modified from a method developed to synthesize N^2-hydroxy-2-amino-3-methylimidazo[4,5-*f*]quinoline (Agus *et al.* 2000). HPLC-MS analysis

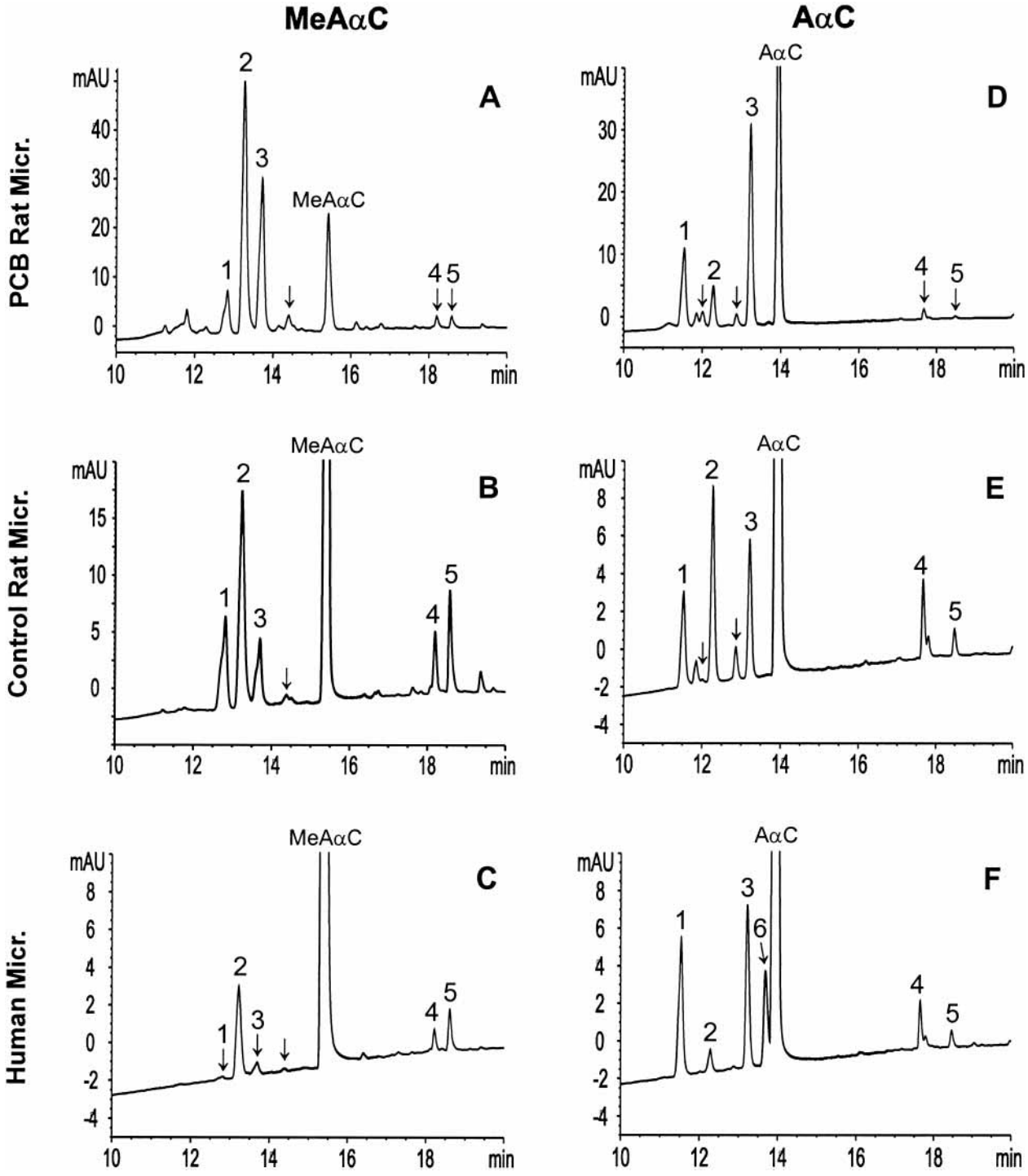

Fig. 2. HPLC-chromatograms monitored at 360 nm showing the metabolism of MeAαC- and AαC in hepatic microsomes (micr.) from PCB-induced rats, control rats and man. Chromatographic conditions are described in Materials and Methods. The chromatograms show MeAαC and its metabolites in hepatic microsomes from PCB-induced rats (A), control rats (B) and man (C), and AαC and its metabolites in hepatic microsomes from PCB-induced rats (D), control rats (E) and man (F). Peaks labelled 1–3 and arrows without labels are detoxified metabolites, while peaks labelled 4–6 indicate activation of the parent compound.

of the synthetic N^2-OH-AαC compound showed retention time, UV spectrum and molecular mass identical to the proposed N^2-OH-AαC metabolite from the microsomal incubation of AαC, showing that some of the AαC was activated to the reactive N^2-OH-compound.

The synthetic N^2-OH-MeAαC has a molecular ion $[M+H]^+$ at *m/z* 214 with a daughter ion $[M+H]^+$ at *m/z* 197 and elute in the HPLC-system at the retentiontime 14.7 min. The presence of a N^2-OH-MeAαC metabolite was not detected in chromatogram A–C (fig. 2).

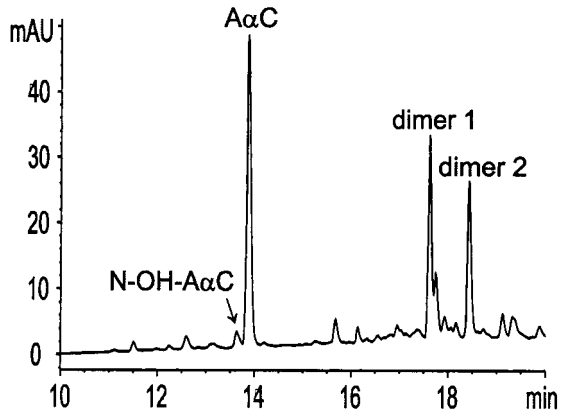

Fig. 3. HPLC-chromatogram monitored at 360 nm showing the synthetic AαC-dimers formed from N-OH-AαC. Chromatographic conditions are described in Materials and Methods.

Addition of acetic anhydride to solutions of N^2-OH-MeAαC and N^2-OH-AαC resulted in formation of three products, each. Fig. 3 shows the HPLC profile of N^2-OH-AαC treated with acetic anhydride. AαC is the major product obtained together with two proposed dimers having identical retention times, UV spectra and mass spectra as the dimers found in the microsomal incubation mixtures. N^2-OH-MeAαC treated with acetic anhydride gave similar results (data not shown). Due to the lack of reactivity of the parent compound, dimers can not be formed from AαC or MeAαC alone. This indicates that the dimers found in the microsomal incubation mixtures are formed from N^2-OH-AαC and N^2-OH-MeAαC, respectively. Further characterisation of the dimers was not attempted.

Metabolic distribution. Fig. 4 shows the percentage distribution of the metabolised part of MeAαC and AαC in the hepatic microsomes from PCB-induced rats, control rats and humans. The calculations are made from liquid scintillation counting data (not shown) on fractions collected from HPLC analysis and dissolved protein precipitates. In the microsomal incubation of [^{3}H]-MeAαC and [^{3}H]-AαC in PCB-induced rat microsomes about 8 and 6%, respectively, of the radioactivity were counted in the protein pellet, indicating activation of the parent compound. In the control rat microsomal incubation of [^{3}H]-MeAαC and [^{3}H]-AαC about 21 and 13%, respectively, of the radioactivity were counted in the protein pellet, and in the human microsomal incubation of [^{3}H]-MeAαC and [^{3}H]-AαC about 9 and 11%, respectively, of the radioactivity were counted in the protein pellet.

Table 1 show the distribution between detoxification and activation of the total amount of metabolised MeAαC and AαC in the three types of microsomes. In PCB-induced rat microsomes about 82 and 92% of the metabolised MeAαC and AαC were detoxified, respectively. In control rat micro-

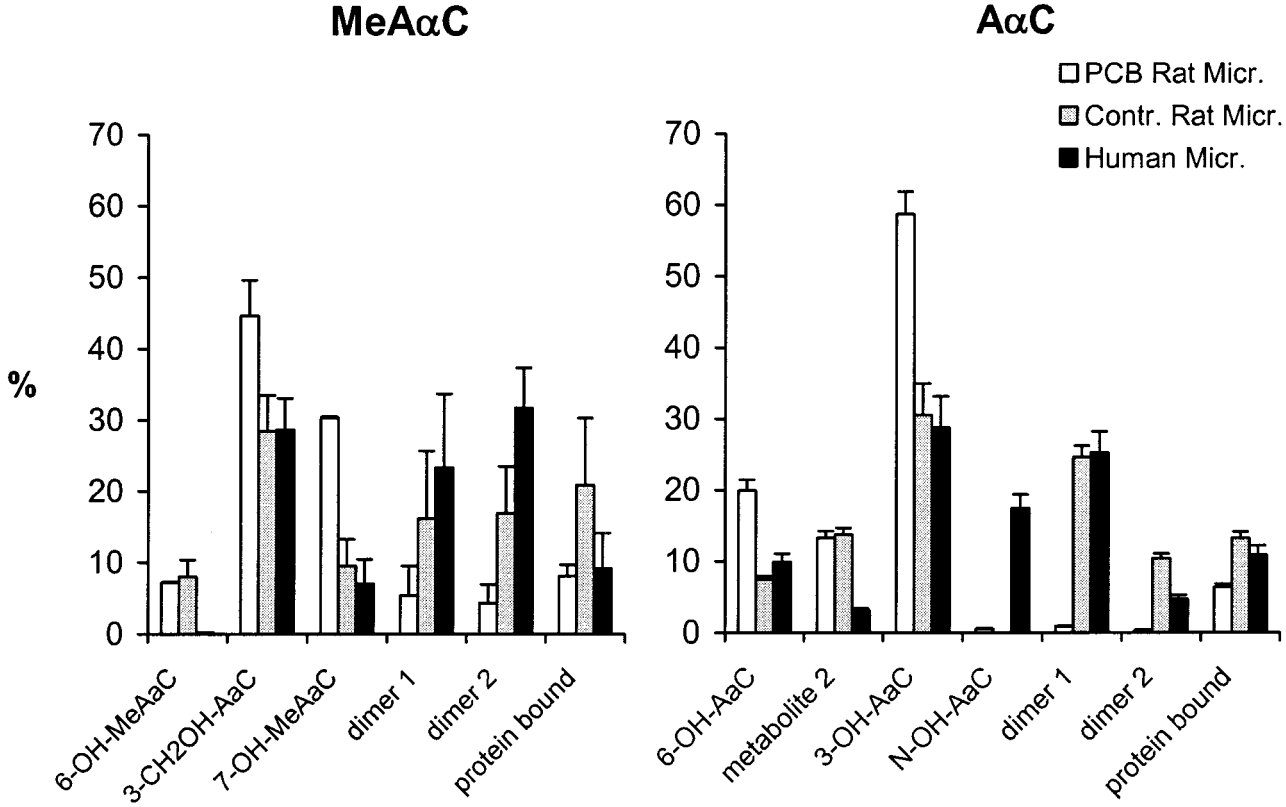

Fig. 4. Distribution of MeAαC- and AαC-metabolites in hepatic microsomes (micr.) from PCB-induced rats, control rats and man shown in percent of the total amount of metabolised parent compound.

Table 1.

Distribution between detoxification and activation of MeAαC- and AαC-metabolites in hepatic microsomes (micr.) from PCB-induced rats, control rats and man. The data are calculated as percent of the total amount of metabolised parent compound.

	Total amount of detoxification and activation (%)		
	PCB rat micr.	Control rat micr.	Human micr.
MeAαC-detoxification	82.2±5.1	46.0±6.5	35.8±1.1
AαC-detoxification	91.8±0.7	51.7±3.1	41.7±2.9
MeAαC-activation	17.8±5.1	54.0±6.5	64.2±1.1
AαC-activation	8.1±0.7	48.3±3.1	58.3±2.9

Values are presented as means±S.D., N=3.

somes about 54 and 48% of the metabolised MeAαC and AαC were activated, and in the human hepatic microsomal incubation 64 and 58% respectively of the metabolised MeAαC and AαC were activated.

Discussion

Using hepatic microsomes from PCB-induced rats, control rats and two different human pools, the *in vitro* metabolism of AαC and MeAαC was investigated. In the microsomal incubation of MeAαC four early-eluting MeAαC-metabolites were found. Three of them are detoxified metabolites, earlier characterized as 6-OH-MeAαC, 3-CH_2OH-AαC and 7-OH-MeAαC (Frandsen *et al.* 1998). A minor metabolite, with the same mass spectrum and a similar UV-spectrum as 6-OH-MeAαC and 7-OH-MeAαC, is probably also a ring-hydroxylated detoxified metabolite of MeAαC.

In our study of AαC metabolism in rat hepatic microsomes we found three major detoxification products and small amounts of two minor metabolites. A previous study of AαC metabolism in hepatic PCB-induced rat microsomes has shown that AαC is metabolised to five metabolites,

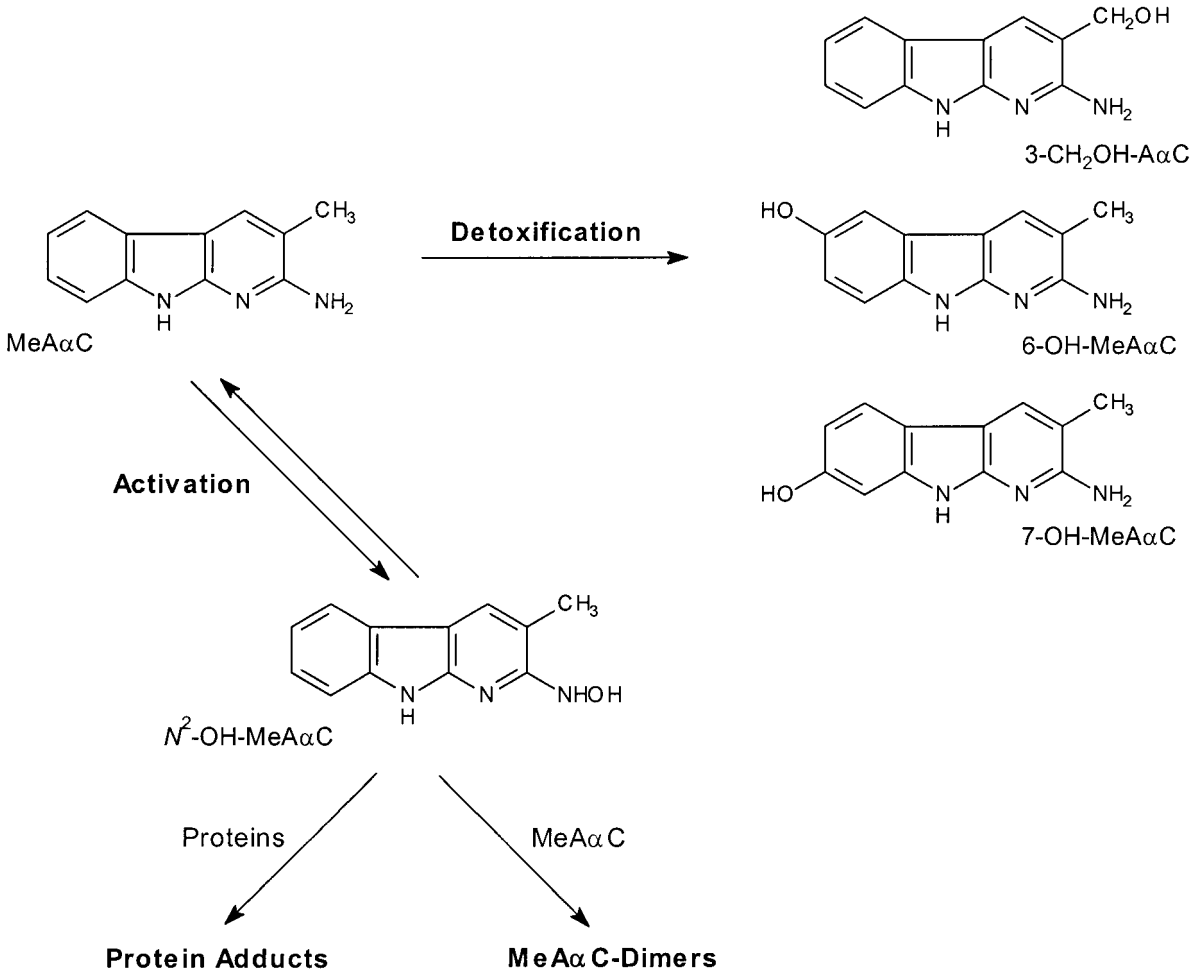

Fig. 5. Metabolic pathway of MeAαC (only the major detoxified metabolites are shown).

three non-mutagenic and two mutagenic metabolites. The two were supposed to be a N^2-hydroxy-derivate and a nitroso-derivate of AαC (Niwa *et al.* 1982). In the human microsomal preparation we found two major detoxification products and one minor, which were identical to the three major products in the rat microsome preparations. In a study of AαC metabolism in human hepatic microsomes, Raza *et al.* (1996) found two major detoxified metabolites characterized as 3-OH-AαC and 6-OH-AαC and four minor metabolites characterized as N^2-OH-AαC, 2-nitroso-AαC and corresponding azoxy- and nitro-derivates. There are good indications that the two major metabolites from the human microsome preparation are 6-OH-AαC and 3-OH-AαC, as characterized by Raza *et al.* (1996). In our microsomal incubations of AαC we did not find nitroso-, azozy- or nitro-derivates of AαC, but we found a much higher amount of N^2-OH-AαC (16%) in the human microsomal incubation.

Another interesting finding was that in all microsomal incubations of both AαC and MeAαC, a considerable amount of the tritium labelled AαC and MeAαC were found in the protein fractions (fig. 4), indicating that these parts of the compounds also had been activated and bound to proteins.

In both the microsomal preparations of AαC and MeAαC, two late-eluting metabolites with molecular masses $[M+H]^+$ at *m/z* 365 and 393, respectively, were found. The same two sets of late-eluting products as well as the parent compounds were obtained, when N^2-OH-AαC and N^2-OH-MeAαC were added acetic anhydride. This indicates that N^2-OH-AαC and N^2-OH-MeAαC were reacting to form two dimers with identical retention times, UV-spectra and masses as found in the HPLC-MS profiles of the microsomal metabolism of AαC and MeAαC. This suggests that the late-eluting AαC and MeAαC metabolites are secondary metabolites, created from the primary and very reactive N^2-OH-derivates and the parent compounds. Fig. 5 shows a suggested metabolic pathway for MeAαC, which is oxidized to three major detoxified metabolites; 3-CH_2OH-AαC, 6-OH-MeAαC and 7-OH-MeAαC, and to the very reactive metabolite N^2-OH-MeAαC. N^2-OH-MeAαC can react in three different ways; reduction back to MeAαC, protein adduct formation, and formation of two MeAαC-dimers with the masses of 392. Our results indicate that the metabolic pathway for AαC seems to be similar, with two major detoxification products, 3-OH-AαC and 6-OH-AαC, and a similar activation/reaction pattern.

It is our experience that N^2-OH-AαC is much more stable than N^2-OH-MeAαC. This could explain why we did not find the primary N^2-OH-MeAαC in the microsomal incubations, but only the secondary metabolites, dimer 1, dimer 2 and the protein bound MeAαC-derivates.

The metabolism of AαC and MeAαC in the three different types of microsomes results in the same metabolites, however in different amounts. In PCB-induced rat microsomes, the major part of the metabolites is detoxified (92 and 82%, respectively), only a little amount is activated (8 and 18%, respectively). A previous study of MeAαC metabolism in PCB-induced hepatic rat microsomes has shown similar results; 83% detoxification and 13% activation (Frandsen *et al.* 1998). In the control rat microsomes there is a nearly fifty-fifty distribution between detoxification and activation, 52% of the metabolised part of AαC was detoxified and 48% was activated, while 46% MeAαC was detoxified and 54% was activated. The major part of the AαC and MeAαC metabolites from the human microsomal incubation were activated, 58 and 64%, respectively. As regards the distribution of the AαC-metabolites in human microsomes, our data are not comparable with a previous study, where Raza *et al.* (1996) found that only 15% of AαC was activated and 85% was detoxified in human hepatic microsomes. In this study, however, binding of the activated metabolite to protein was not taken into consideration. *In vitro* studies of another heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has shown species variation in metabolic distribution similar to our results on AαC and MeAαC. The ratio of *N*-hydroxylation to the non genotoxic 4'-hydroxylated derivate of PhIP was 97:1, 3.3:1 and 1.7:1 for humans, rats and mice, respectively (Lin *et al.* 1995).

Conclusion. In this study we have investigated the *in vitro* metabolism of the α-carbolines, AαC and MeAαC in human and rat hepatic microsomes. We have synthesised N^2-OH-derivates and dimers of both AαC and MeAαC for comparison with the activated metabolites found in the microsomal incubations. We have revised the methods for the synthesis of the N^2-OH-derivates, and our HPLC-MS method is specially developed to conserve the activated metabolites of the α-carbolines.

Our data show that AαC and MeAαC have similar metabolic patterns. The results also show a remarkable difference in the metabolic pattern of the α-carbolines in human hepatic microsomes, in control rat microsomes and in PCB-induced rat microsomes respectively. In human hepatic microsomes, a major part of the α-carbolines are metabolically activated, in hepatic microsomes from control rats there is a fifty-fifty distribution between activation and detoxification and in PCB-induced hepatic microsomes the α-carbolines are mainly detoxified.

In the light of these results we have proposed a new *in vitro* metabolic pathway for both AαC and MeAαC. Finally it is concluded that, due to the higher degree of activation in human microsomes, human exposure to AαC and MeAαC may result in a higher cancer risk than estimations based on long-term cancer experiment in rodents.

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References

Agus, C., K. F. Ilett, F. F. Kadlubar & R. F. Minchin: Characterization of an ATP-dependent pathway of activation for the heterocyclic amine carcinogen N-hydroxy-2-amino-3-methylimidazo[4, 5-f]quinoline. *Carcinogenesis* 2000, **21**, 1213–1219.

Eisenbrand, G. & W. Tang: Food-borne heterocyclic amines. Chemistry, formation, occurrence and biological activities. A literature review. *Toxicology* 1993, **84**, 1–82.

Frandsen, H., S. Damkjær, S. Grivas, R. Andersson, M. L. Binderup & J. C. Larsen: Microsomal metabolism and activation of the environmental carcinogen 2-amino-3-methyl-9H-pyrido[23-b]indole. *Mutagenesis* 1998, **13**, 181–185.

Frandsen, H., P. A. Nielsen, S. Grivas & J. C. Larsen: Microsomal metabolism of the food mutagen 2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]-quinoxaline to mutagenic metabolites. *Mutagenesis* 1994, **9**, 59–65.

Gross, G. A. & A. Gruter: Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. *J. Chromatogr.* 1992, **592**, 271–278.

Hasegawa, R.: Dose-dependent formation of preneoplastic foci and DNA adducts in rat liver with 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeA alpha C) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis* 1992, **13**, 1427–1431.

Holder, C. L., S. W. Preece, S. C. Conway, Y. M. Pu & D. R. Doerge: Quantification of heterocyclic amine carcinogens in cooked meats using isotope dilution liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 1997, **11**, 1667–1672.

Holme, J. A., H. Wallin, G. Brunborg, E. J. Søderlund, J. K. Hongslo & J. Alexander: Genotoxicity of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP): formation of 2-hydroxamino-PhIP, a directly acting genotoxic metabolite. *Carcinogenesis* 1989, **10**, 1389–1396.

King, R. S., C. H. Teitel & F. F. Kadlubar: *In vitro* bioactivation of N-hydroxy-2-amino-alpha-carboline. *Carcinogenesis* 2000, **21**, 1347–1354.

Lin, D. X., N. P. Lang & F. F. Kadlubar: Species differences in the biotransformation of the food-borne carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine by hepatic microsomes and cytosols from humans, rats, and mice. *Drug Metab Disp.* 1995, **23**, 518–524.

Manabe, S., O. Wada & Y. Kanai: Simultaneous determination of amino-alpha-carbolines and amino-gamma-carbolines in cigarette smoke condensate by high-performance liquid chromatography. *J Chromatogr.* 1990, **529**, 125–133.

Matsumoto, T., D. Yoshida & H. Tomita: Determination of mutagens, amino-alpha-carbolines in grilled foods and cigarette smoke condensate. *Cancer Lett.* 1981, **12**, 105–110.

Nagao, M., Y. Fujita, K. Wakabayashi & T. Sugimura: Ultimate forms of mutagenic and carcinogenic heterocyclic amines produced by pyrolysis. *Biochem Biophys. Res. Commun.* 1983, **114**, 626–631.

Niwa, T., Y. Yamazoe & R. Kato: Metabolic activation of 2-amino-9H-pyrido[2,3-b]indole by rat-liver microsomes. *Mutat. Res.* 1982, **95**, 159–170.

Ohgaki, H., N. Matsukura, K. Morino, T. Kawachi, T. Sugimura & S. Takayama: Carcinogenicity in mice of mutagenic compounds from glutamic acid and soybean globulin pyrolysates. *Carcinogenesis* 1984, **5**, 815–819.

Pfau, W., U. Brockstedt, C. Schulze, G. Neurath & H. Marquardt: Characterization of the major DNA adduct formed by the food mutagen 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAalphaC) in primary rat hepatocytes. *Carcinogenesis* 1996, **17**, 2727–2732.

Pfau, W., C. Schulze, T. Shirai, R. Hasegawa & U. Brockstedt: Identification of the major hepatic DNA adduct formed by the food mutagen 2-amino-9H-pyrido[2,3-b]indole (A alpha C). *Chem. Res. Toxicol.* 1997, **10**, 1192–1197.

Raza, H., R. S. King, R. B. Squires, F. P. Guengerich, D. W. Miller, J. P. Freeman, N. P. Lang & F. F. Kadlubar: Metabolism of 2-amino-alpha-carboline. A food-borne heterocyclic amine mutagen and carcinogen by human and rodent liver microsomes and by human cytochrome P4501A2. *Drug Metab. Disp.* 1996, **24**, 395–400.

Richling, E., C. Decker, D. Haring, M. Herderich & P. Schreier: Analysis of heterocyclic aromatic amines in wine by high-performance liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* 1997, **791**, 71–77.

Skog, K., A. Solyakov, P. Arvidsson & M. Jägerstad: Analysis of nonpolar heterocyclic amines in cooked foods and meat extracts using gas chromatography-mass spectrometry. *J Chromatogr. A* 1998, **803**, 227–233.

Solyakov, A., K. Skog & M. Jägerstad: Heterocyclic amines in process flavours, process flavour ingredients, bouillon concentrates and a pan residue. *Food Chem. Toxicol* 1999, **37**, 1–11.

Sugimura, T.: Carcinogenicity of mutagenic heterocyclic amines formed during the cooking process. *Mutat. Res.* 1985, **150**, 33–41.

Sugimura, T., M. Nagao & K. Wakabayashi: Carcinogenicity of food mutagens. *Environ. Health Perspect.* 1996, **104, Suppl 3**, 429–433.

Wakabayashi, K.: Identification of new mutagenic heterocyclic amines and quantification of known heterocyclic amines. *Princess Takamatsu Symp.* 1995, **23**, 39–49.

Wild, D., B. E. Watkins & M. Vanderlaan: Azido- and nitro-PhIP, relatives of the heterocyclic arylamine and food mutagen PhIP–mechanism of their mutagenicity in Salmonella. *Carcinogenesis* 1991, **12**, 1091–1096.

Yoshida, D. & T. Matsumoto: Amino-alpha-carbolines as mutagenic agents in cigarette smoke condensate. *Cancer Lett.* 1980, **10**, 141–149.