Biochemical Pharmacology, Vol. 38, No. 2, pp. 279–287, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

COVALENT BINDING OF 4,4′-METHYLENEBIS-(2-CHLOROANILINE) TO RAT LIVER DNA *IN VIVO* AND OF ITS *N*-HYDROXYLATED DERIVATIVE TO DNA *IN VITRO* *

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(*Received* 17 *May* 1988; *accepted* 16 *August* 1988)

Abstract—The binding of [ring-^{3}H]4,4′-methylenebis(2-chloroaniline) (MOCA) to rat liver DNA following i.p. injection is demonstrated. Three discrete adducts were eluted on HPLC following enzymic hydrolysis to the nucleoside level. Three adducts, with the same retention times on HPLC, were present after i.p. injection of the *N*-acetyl derivative of MOCA tritiated in the benzene rings. Only two of these adducts were found when the *N*-acetyl derivative, tritiated on the acetyl group, was used. Thus, at least one of the adducts formed by MOCA is not acetylated.

The *N*-hydroxy derivative of MOCA was synthesised and reacted with DNA *in vitro*. Following enzymic hydrolysis of this DNA, the major product was shown to co-elute with the radiolabelled non-acetylated adduct produced in the liver DNA of animals injected with [ring-^{3}H]MOCA. This same compound was also isolated following the reaction of *N*-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA, and subsequent enzymic hydrolysis. The NMR and mass spectra of the synthetic adduct were consistent with *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol. Thus, the major adduct formed *in vivo* has involved cleavage of the bond between the methylene bridge and one of the aromatic nuclei of MOCA.

4,4′-Methylenebis(2-chloroaniline)‖ is an aromatic diamine used as a curing agent in the plastics industry. It is carcinogenic in a variety of laboratory animal species, inducing tumours of the lung, liver and mammary gland in rats [1–3], of the liver in mice [2] and of the bladder in dogs [4]. In man, it has not been shown to be carcinogenic, due rather to the lack of any properly controlled study [5] than there being conclusive negative data. However, since a variety of aromatic amines are known to induce bladder cancer in man, e.g. 2-naphthylamine, 4-aminobiphenyl and benzidine, investigations into the potential hazards of MOCA are warranted.

From studies on the metabolism and nucleic acid binding of a number of aromatic amines, it has been shown that *N*-hydroxylation occurs as the first step in the activation pathway [6]. In the case of benzidine, *N*′-acetylation is required to stabilise the molecule prior to *N*-hydroxylation [7]. Very little has been published on the metabolic fate of MOCA

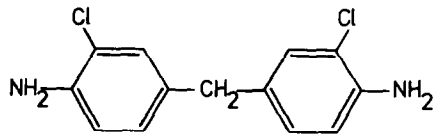

Fig. 1. The molecular structure of MOCA.

(whose structure is shown in Fig. 1). A number of metabolites have been isolated [8] and, whilst some have been proposed as active intermediates in the carcinogenic process [9, 10], only a limited amount of work was done to demonstrate this. The aim of the present study was to elucidate at least part of the metabolic pathway by which MOCA is activated in rat liver to a DNA-binding species. Further, it was of interest to determine the extent and nature of any covalent interaction between MOCA and DNA. The often quoted similarity of the molecular structure of MOCA to benzidine (e.g. Refs 9 and 11) lead us to investigate the role played by cell mediated *N*-acetylation. This has been shown to be a pre-requisite for binding of benzidine to rat liver DNA [7].

MATERIALS AND METHODS

Animals. Rats (150–200 g) were males of a Wistar-derived strain obtained from Bantin and Kingman Ltd, Hull, U.K. Animals were fed on modified Diet 41B (Oxoid Ltd, London, U.K.) and given water *ad libitum*. During the experimental period, animals were housed in a glass metabolism cage with a continuous supply of food and water.

* This work was supported by a grant from the Health and Safety Executive.

‖ Abbreviations used: MOCA, 4,4′-methylenebis(2-chloroaniline); AcMOCA, *N*-acetyl-4,4′-methylenebis(2-chloroaniline); MOCA-NO_2, 3,3′-dichloro-4-nitro-4′-amino-diphenylmethane; MOCA-NHOH, *N*-hydroxy-4,4′-methylenebis(2-chloroaniline); bis tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; DMSO, dimethylsulphoxide; DMF, dimethylformamide; HPLC, high performance liquid chromatography; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; i.p., intraperitoneal; SD, standard deviation.

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The following reagents were purchased from the sources indicated. DNase 1 (type III), nuclease P_1, alkaline phosphatase (type III-S), acid phosphatase (type I), calf thymus DNA (type I), and neutralised charcoal, Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.); phosphodiesterase (from *Crotalus durissus*), Boehringer Mannheim (Lewes, East Sussex, U.K.); MOCA, Hickson and Welch (Castleford, U.K.). This was purified by recrystallisation from methanol/water. [^{3}H]Acetic anhydride (stated specific activity 100 mCi/mmol), New England Nuclear (Southampton, U.K.); hydroxylapatite (fast flow), Calbiochem (Cambridge, U.K.); 3-chlorobenzyl chloride (98%), Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.); 2-chloroaniline, Koch Light Ltd. (Haverhill, Suffolk, U.K.); 1,4-dioxan (slr grade) and Optiphase "MP", Fisons (Loughborough, Leicestershire, U.K.); silica gel for column chromatography (type 60, 0.063–0.200 mm) and the TLC (type 60, GF_{254}), Merck (Darmstadt, F.R.G.); preformed TLC plates (Polygram silG (0.25 mm silica gel without gypsum)), Camlab (Cambridge, U.K.); and Spherisorb ODS-2, Phase Sep. (Queensferry, Clwyd, U.K.). All other chemicals used were of analytical grade; butan-1-ol was further purified by distillation.

Synthesis of radiochemicals. [Ring-^{3}H]MOCA (specific activities 27.0 and 56.2 mCi/mmol) was prepared by tritium exchange of MOCA (by Dr. J. R. Jones, University of Surrey). Tritium NMR showed the compound to be ring labelled (single peak, 6.8), whilst reversed-phase HPLC (using a water: methanol gradient) and TLC (on silica, solvent systems benzene:petroleum ether (60–80):methanol, 5 vol.:5 vol.:1 vol., and acetone:benzene:cyclohexane, 13 vol.:15 vol.:13 vol.) showed a radiochemical purity of 96.7%.

[Acetyl-^{3}H]AcMOCA was made by adding [^{3}H]acetic anhydride (stated specific activity 100 mCi/mmol, 235 μl, 20% in benzene, 0.5 mmol) to MOCA (0.761 g, 2.85 mmol) dissolved in a mixture of benzene (10 ml) and diethylether (5 ml). After stirring for 60 min at room temperature, a solution of sodium carbonate (0.05 M, 10 ml) was added and, after mixing, the organic layer was removed. The aqueous solution was re-extracted with diethylether (10 ml) and the combined organic samples were evaporated to dryness. The residue was dissolved in a minimum volume of benzene and separated on a silica column eluted with benzene: petroleum ether (60–80):methanol (5 vol.:5 vol.: 1 vol.). Fractions containing [acetyl-^{3}H]AcMOCA were pooled and subsequent analysis by reversed-phase HPLC (using a water:methanol gradient) and TLC on silica (solvent system acetone:benzene: cyclohexane, 13 vol.:15 vol.:13 vol.) showed a radiochemical purity of 97.2% (specific activity 35.5 mCi/mmol: yield 68%).

[Ring-^{3}H]AcMOCA (specific activity 25.4 mCi/mmol) was made in a similar manner, by adding acetic anhydride (11 μl, in 100 μl benzene, 0.12 mmol) to [ring-^{3}H]MOCA (27 mCi/mmol: 20 mCi, 0.74 mmol). After reaction and purification as above, the radiochemical purity was found to be 92% (as determined by HPLC and TLC) with only 1% of the impurity present as [ring-^{3}H]MOCA.

Non-radiolabelled AcMOCA was synthesised in a similar manner and its structure confirmed by NMR (2.20 (singlet, 3H, CH_3), 3.60 (singlet, 2H, NH_2), 3.75 (singlet, 2H, CH_2), 6.60–7.20 (multiplet, 5H, aromatic), 7.55 (singlet, 1H, NH), 8.20 (doublet, 1H, aromatic-H^6). $CDCl_3$ solvent) and mass spectroscopy (m/e, 308/310/312 (M); 273/275 (M-Cl); 266/268/270 (M-CH_2CO); 167/169 (M-Ch(Cl)(NH_2)(CH_3)). This standard was shown to co-chromatograph with both radiolabelled preparations on TLC and HPLC.

Synthesis of MOCA–NHOH. (i) 2-Chloroaniline (17.4 ml, 0.17 mol) was added to a mix of water (300 ml) and hydrochloric acid (14 ml) with stirring. After dissolution, charcoal (3 g) was added and the solution heated to 50° for 5 min, with stirring. The charcoal was removed by filtration and to the cooled solution was added acetic anhydride (20 ml, 0.21 mol) followed by sodium acetate (25 g of the trihydrate, 0.18 mol, dissolved in 75 ml water). The solution was stirred vigorously and placed on ice. A precipitate of 2-chloroacetanilide was formed on stirring and after no more precipitate was formed, the solid was filtered, washed with water and left to air dry. Yield 18.8 g (67%). (NMR: 2.20 (singlet, 3H, CH_3), 6.90–7.40 (multiplet, 3H, aromatic), 7.70 (singlet, 1H, NH), 8.25 (doublet, 1H, aromatic-H^6). $CDCl_3$ solvent).

(ii) 3-Chloro-4-nitrobenzyl chloride was prepared by nitration of 3-chlorobenzyl chloride [12].

(iii) 2-Chloroacetanilide (10.8 g, 0.064 mol) was dissolved with warming in nitromethane (15 ml). To this was added aluminium chloride (0.9 g, 0.0067 mol, dissolved in nitromethane (1 ml)) and 3-chloro-4-nitrobenzyl (1.30 g, 0.0063 mol). The mixture was heated under reflux for 72 hr, during which time considerable precipitation occurred. After cooling and further addition of nitromethane (10–20 ml), the mixture was poured onto ice (30 g) and hydrochloric acid (50 ml). The organic layer was removed, washed twice with water and evaporated to dryness.

(iv) Sulphuric acid (70% (w/v), 40 ml) was added to the residue and heated under reflux for 30 min. After cooling, the mixture was neutralised (NaOH) and extracted twice with chloroform. The chloroform was removed by evaporation and 2-chloroaniline was removed by washing the residue once with 20% (v/v) HCl (100 ml) and twice with 10% (v/v) HCl (100 ml), followed by two washes with water. The residue was extracted with ether which, after removal, left a brown residue of MOCA-NO_2 (typically 0.1 g).

The reaction was monitored by reversed-phase HPLC (water:methanol gradient; 30% methanol 10 min, 30–70% over 40 min, 70–100% over 5 min; flow rate 2 ml/min). Usually only a single UV absorbing peak (260 nm) was seen, but occasionally a small amount of 3-chloro-4-nitrobenzyl chloride was present. This was removed by dissolving the residue in 1,4-dioxan (1 ml per 10 mg residue) followed by addition of an equal volume of sodium hydroxide solution (0.2 M) [13]. After 2.5 hr the solution was neutralised by addition of the appropriate amount of HCl (1 M solution) and, after centrifugation (2800 rpm, 10 min on a bench centrifuge (Centaur 2, MSE Scientific Instruments, Crawley, U.K.)), the

clear yellow supernatant was recovered and evaporated to dryness, leaving a preparation of MOCA-NO_2 which showed a single UV absorbing peak on HPLC. (Mass spectroscopy showed m/e 296/298/300 (M), 250/252/254 (M-NO_2), 237/239/241 (unknown), 170/172 (chloronitrobenzyl ion), 167 (unknown), 140/142 (aminochlorobenzyl ion). Elemental analysis: C-54.51, H-3.89, N-8.78, Cl-24.17 (calculated; C-52.52, H-3.37, N-9.43, Cl-23.91). A sample of MOCA-NO_2 reduced with tin and hydrochloric acid co-chromatographed with an authentic sample of MOCA on TLC and HPLC).

(v) To further ensure purity, a sample of MOCA-NO_2 (100 mg) was dissolved in a mixture of benzene: petroleum ether 60–80:methanol (5 vol.:5 vol.:1 vol.; 2 ml) and eluted from a silica column with the same mixture. Fractions containing MOCA-NO_2 were pooled and the solvent was removed by evaporation. (18.5 mg, 62 μmol) of this sample was dissolved in DMF (745 μl) and cooled on ice. To this was added ammonium chloride solution (1 M, 248 μl) and the resulting solution was saturated with argon whilst still on ice. Acid-washed zinc (12.3 mg) was added in 2 portions, 5 min apart, and the solution was then left on ice with a slow stream of argon passing through it. After 4 hr, the solution was extracted twice with ice-cold argon-saturated dichloromethane (cold argon-saturated water had to be added after the first extraction) and the combined organic extracts were washed with water (ice-cold and argon-saturated). The dichloromethane was removed by evaporation with argon and the MOCA-NHOH was either stored at −90° or redissolved in DMF (150 μl) and reacted immediately with DNA. The course of the reaction was monitored (hourly) by HPLC; after 4 hr, essentially all of the MOCA-NO_2 was converted into MOCA-NHOH, whilst if the reaction was left longer, unknown products were formed, as determined by HPLC. HPLC conditions were as described in (iv) above. Under these conditions, MOCA-NO_2 was eluted after 52 min, MOCA-NHOH after 46 min and MOCA after 42 min. AcMOCA is eluted after 38 min. Mass spectroscopy of MOCA-NHOH gave m/e 282/284/286 (M), 266/268/270 (M-O), 237/239/241 (unknown), 167 (unknown), 140/142 (aminochlorobenzyl ion).

Synthesis of N-hydroxy-4-amino-3-chlorobenzyl alcohol. (i) 3-Chloro-4-nitrobenzyl alcohol was initially prepared by acid hydrolysis of 3-chloro-4-nitrobenzyl chloride but a more satisfactory method was subsequently used [12].

(ii) 3-Chloro-4-nitrobenzyl alcohol could be reduced to the *N*-hydroxy derivative using the same conditions as for MOCA-NO_2. The preferred method, however, (giving greater yields) was to dissolve 3-chloro-4-nitrobenzyl alcohol (0.35 g, 1.87 mmol) in ethanol (25 ml), to which water (25 ml) and ammonium chloride solution (1 M, 10 ml) were added. After cooling on ice and degassing with argon, acid-washed zinc (0.4 g, 6.12 mmol) was added in two portions, 5 min apart. The solution was left stirring with a stream of argon passing through it and the reaction was monitored by HPLC. Using the same gradient conditions as described above, 3-chloro-4-nitrobenzyl alcohol eluted at 30 min, the amine after 5 min and the N-OH derivative after 3.5 min. When all the 3-chloro-4-nitrobenzyl alcohol had been reduced (usually after approximately 4 hr), water (25 ml) and dichloromethane (25 ml) were added to the reaction mixture. After mixing, the organic layer was removed and washed with water (25 ml). The aqueous phases were then combined and extracted with an equal volume of ethyl acetate. The ethyl acetate was removed in a stream of argon to leave *N*-hydroxy-4-amino-3-chlorobenzyl alcohol (yield 0.13 g, 40%). The product contained less than 10% amine (and dimeric products [12] as determined by HPLC). Mass spectroscopy gave m/e 173/5 (M), 157/9 (M-O), 156/8 (M-OH), 143/5 (M-NO or M-HCHO), 140/2 (M-OH-O), 93 (unknown).

In vivo *experiments.* Rats were injected i.p. with 1 mCi of [ring-^{3}H]MOCA (89 μmol/kg), [acetyl-^{3}H]AcMOCA (141 μmol/kg) or [ring-^{3}H]AcMOCA (197 μmol/kg) dissolved in DMSO (0.2 ml), to achieve equivalent doses of radiolabel. After 24 hr animals were killed, their livers removed and DNA isolated and purified by solvent extraction and hydroxylapatite column chromatography, as previously described [14].

In vitro *experiments.* Rat liver slices were prepared as previously described [15]. To each of the solutions (usually 12) containing approximately 500 mg each of liver slices was added 0.89 μmol [ring-^{3}H]MOCA or 1.40 μmol [acetyl-^{3}H]AcMOCA dissolved in 50 μl DMSO (i.e. 50 μCi/liver slice incubation). Following a 2.5 hr incubation at 37° in an oxygen atmosphere, the liver slices were combined, sedimented by centrifugation and DNA was extracted as referenced above.

Adduct analysis. Purified DNA was redissolved in bis tris (5 mM, pH 7.1) to give a final concentration of approximately 1 mg/ml. (At this stage, the amount of DNA was determined [16]. Where appropriate, DNA was counted for radioactivity.) The DNA was enzymically hydrolysed to the nucleoside level and the modified nucleosides were recovered by extraction into butan-1-ol. The procedure was as described in [7]. However, for hydrolysis, phosphodiesterase was also added at a concentration of 0.072 units/mg DNA after the 3 hr incubation with DNase I. After removal of the butan-1-ol by rotary evaporation, the residue was redissolved in 30% (v/v) methanol and analysed by reversed-phase HPLC using a flow rate of 2 ml/min and gradient conditions as described in Fig. 2. Fractions (1 ml) were collected and radioactivity determined by scintillation counting.

Reaction of MOCA-NHOH with DNA. MOCA-NHOH prepared as described above and dissolved in 150 μl of DMF, was added to a solution of DNA (30 mg dissolved in a 8 ml solution containing citric acid (0.01 M) and EDTA (1 mM), pH 4.6 (by addition of potassium hydroxide)) saturated with argon and, after mixing, was incubated at 37°. After 24 hr, the mixture was extracted with an equal volume of water-saturated butan-1-ol. The DNA was ethanol-precipitated and washed and dried in acetone. The DNA was redissolved in bis tris buffer and enzymically hydrolysed as referenced for *in vivo*-derived samples. Butan-1-ol soluble modified nucleosides were analysed by reversed-phase HPLC.

In one case, following the reduction of MOCA-NO_2 (5 mg), the MOCA-NHOH product was col-

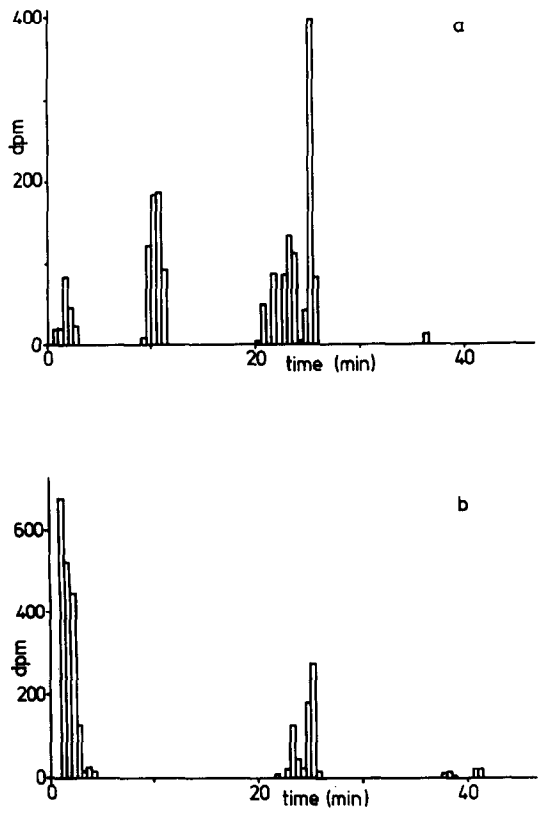

Fig. 2. HPLC profile of radioactivity associated with rat liver DNA from animals injected i.p. with (a) 89 μmol/kg [ring-^{3}H]MOCA, or (b) 141 μmol/kg [acetyl-^{3}H]AcMOCA, and killed 24 hr following treatment. Liver DNA was isolated, enzymically hydrolysed and the adducts partitioned into butan-1-ol. The ODS-2 reversed phase column (250 mm × 4.6 mm) was eluted with a water: methanol gradient; 30% methanol for 10 min, 30%–70% over 40 min, 70%–100% over 5 min, 100% for 5 min, flow rate 2 ml/min. 1 ml fractions were collected and assayed for radioactivity.

lected from reversed-phase HPLC runs (using a shorter gradient of: 30% methanol for 5 min, then 30%–100% methanol over 25 min). Methanol was removed by evaporation and the aqueous suspension was immediately reacted with DNA as described above. A sample of evaporated MOCA-NHOH was analysed on HPLC, conditions as described in Fig. 2, and only a single UV absorbing peak was detected.

Large scale reaction of MOCA-NHOH and DNA was performed in order to produce modified DNA in sufficient quantity for structural characterisation following enzyme hydrolysis.

Reaction of N-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA. *N*-Hydroxy-4-amino-3-chlorobenzyl alcohol (0.13, 0.75 mmol) was dissolved in DMF (1.1 ml) and added to a solution of DNA (315 mg in citrate buffer (70 ml)). After reacting for 24 hr at 37°, the DNA was isolated and hydrolysed as described and modified nucleosides were analysed by reversed-phase HPLC.

Silylation. The adduct eluting at 10 min on reversed phase HPLC was silylated by reaction with an excess of pyridine:bis(trimethyl-silyl)trifluoroacetamide (BSTFA) (1:1) for 4 hr at 37°. For fast atom bombardment mass spectroscopy the adduct was reacted at room temperature for 10 min with BSTFA:methyl trimethlylsilyl trifluoroacetamide:DMA (1:1:1).

Instrumentation. Mass spectra were obtained on an AEI MS 30, with electron impact at 70 eV, and in the fast atom bombardment mode on a Kratos MS-50. FAB was by xenon atoms 10 keV acceleration. The silylated sample was dissolved in DMF and introduced into a matrix of thioglycerol. The analyses were performed with a 6 keV accelerating voltage. Resolution was 3000 and the scan rate was a linear scan from 2000 to 150 mass units over 30 sec. NMR spectra at 90 MHz were obtained on a Jeol FX90Q, with the exception of the adduct, which was obtained on a Bruker 360 machine. HPLC was on a Pye-Unicam PU 4000 series double pump system. Radioactivity was determined following addition of 4 ml Optiphase scintillant to 1 ml sample in a Packard Tricarb-300 scintillation counter.

RESULTS

DNA binding

Twenty-four hours after i.p. injection of tritiated MOCA and AcMOCA (as described in Materials and Methods), rat liver DNA was isolated, purified and analysed to determine the amount of covalently bound radioactivity. Values obtained were [ring-^{3}H]MOCA 9.2 ± 4.1 pmol/mg DNA, [acetyl-^{3}H]AcMOCA 5.3 ± 2.0 pmol/mg DNA (values ± SD from at least three separate experiments) and [ring-^{3}H]AcMOCA 8.0 pmol/mg DNA (variance less than 2.5% from two separate experiments). Similarly with rat liver slices, radioactivity was found bound to isolated, purified DNA following incubation with [ring-^{3}H]MOCA (binding 20.7 ± 8.4 pmol/mg DNA) and [acetyl-^{3}H]AcMOCA (binding 15.6 ± 1.3 pmol/mg DNA).

In vivo *adduct analysis*

Butanolic extracts of enzymically hydrolysed DNA samples from i.p. injected rats were analysed by reversed-phase HPLC. Fractions were collected and counted for radioactivity and representative profiles are shown in Fig. 2. Hydrolysed [ring-^{3}H]MOCA-DNA consistently resulted in resolution of three reproducible peaks of radioactivity which appeared in all analyses performed. These eluted at 10, 23.5 and 25.5 min (Fig. 2a). When hydrolysed [acetyl-^{3}H]AcMOCA-DNA was applied to HPLC, two reproducible peaks were resolved which eluted at 23.5 and 25.5 min (Fig. 2b). When [ring-^{3}H]AcMOCA samples were analysed, the same profile as from [ring-^{3}H]MOCA was seen. Thus the early running peak was present when AcMOCA was ring labelled but not detected when labelled in the acetyl moiety, showing it to contain the aromatic nucleus of MOCA but not the *N*-acetyl function.

Liver slice DNA adduct analysis

Freshly prepared liver slices were incubated under oxygen with [ring-^{3}H]MOCA. DNA was purified and, after hydrolysis, was analysed by HPLC. The

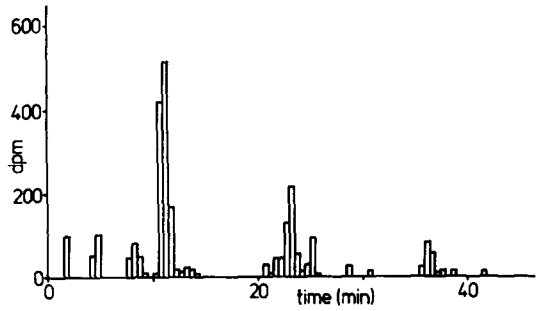

Fig. 3. HPLC profile of radioactivity associated with rat liver DNA following incubation of liver slices (0.4–0.6 g) with 0.89 μmol [ring-^{3}H]MOCA. Modified DNA was isolated and analysed by reversed-phase HPLC, as described in Fig. 2.

adduct profiles are shown in Fig. 3. It can be seen that there is essentially no qualitative difference between the profile of MOCA-reacted DNA from liver slices (Fig. 3) or from *in vivo* samples (Fig. 2a). (The additional, early eluting peaks (between 5 and 10 min) were always observed from liver slice samples, but only as minor constituents.)

Analysis of adducts formed between DNA and MOCA-NHOH

MOCA-NHOH was synthesised and extracted into dichloromethane, the solvent was removed and the residue redissolved in DMF immediately prior to reaction with DNA and the purity confirmed by HPLC. The reaction with DNA was carried out at pH 4.6 [17]. Under these conditions the aromatic hydroxylamine reacted directly with DNA during a 24-hr incubation period. The hydrolysed DNA was analysed by HPLC and two UV-absorbing peaks were observed at 10 min and 23.5 min. When co-injected with a sample of hydrolysed DNA obtained from liver slices incubated with [ring-^{3}H]MOCA, the two UV absorbing peaks exactly co-eluted with the first two radiolabelled peaks. This is shown in Fig. 4 (where the two peaks are designated I and II respectively). Peak I was collected and re-injected using an extended water:methanol gradient. The radioactivity remained exactly associated with the UV absorbing material (Fig. 5) confirming the identical chromatographic properties of the adduct in this system.

A sample of MOCA-NO_2 was purified on silica and reduced to MOCA-NHOH, which was then reacted with DNA. Similarly, a sample of MOCA-NHOH was purified on HPLC and reacted with DNA. In both of these cases, the HPLC profiles of modified nucleosides were identical to that shown in Fig. 4.

Following a large-scale reaction between MOCA-NHOH and DNA, the modified DNA was enzymically hydrolysed and adduct I was purified by multiple injection on HPLC, with a final yield of approximately 200 μg. This sample was analysed by NMR spectroscopy using Fourier Transform analysis following irradiation of the sample, in d_6-DMSO, at

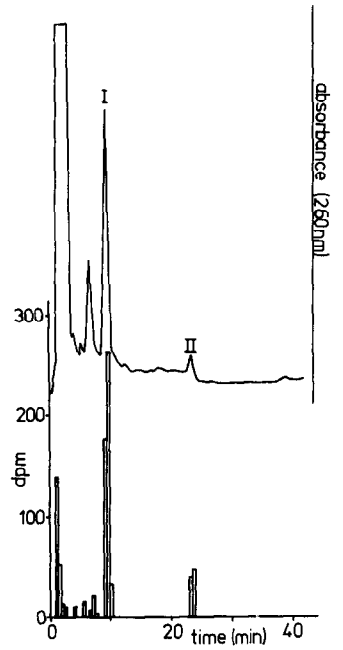

Fig. 4. HPLC profile of enzymically hydrolysed DNA following reaction with MOCA-NHOH for 24 hr at 37°. Modified DNA was partitioned into butan-1-ol and co-injected with modified DNA obtained following incubation of [ring-^{3}H]MOCA with rat liver slices. HPLC conditions were the same as described in Fig. 2.

360 MHz in a Bruker machine. Details are shown in Table 1.

Analysis of adducts formed between N-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA

Reduction of 3-chloro-4-nitrobenzyl alcohol with zinc and ammonium chloride gave the *N*-hydroxy

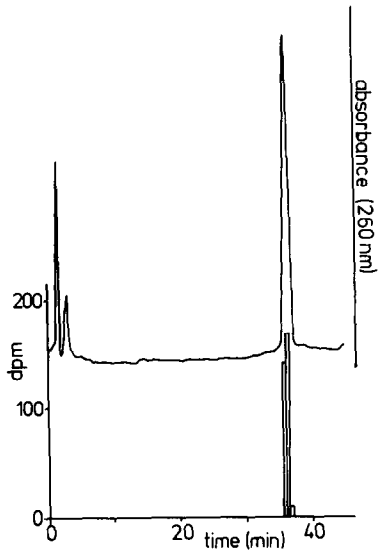

Fig. 5. HPLC profile of adduct I, isolated as described in Fig. 4 and re-chromatographed; water:methanol gradient was 10% methanol for 10 min, 10%–30% over 20 min, 30% for 10 min.

Table 1. Assignment of protons in NMR spectrum of adduct I

Chemical shift*	Effect of D_2O†	Assignment‡
7.9 (s, 1H)	—	C-2, H(Ad)
7.2 (d, 1H, $J = 2H_z$)	—	C-2, H(Ar)
7.0 (dd, 1H, $J = 8H_z$, $2H_z$)	—	C-6, H(Ar)
6.9 (br s, 1H)	disappears	C-4, N-H(Ar)
6.7 (d, 1H, $J = 8H_z$)	—	C-5, H(Ar)
6.1 (t, 1H, $J = 7H_z$)	—	C-1, H(R)
5.2 (m, 3H)	all disappear	C-3, OH(R) and C-6, NH_2(Ad)
4.9 (br s, 1H)	disappears	C-5, OH(R)
4.4 (m, 1H)	simplifies	C-3, H(R)
4.3 (d, 2H, $J = 5H_z$)	doublet→ singlet	C-1, CH_2(Ar)
3.8 (m, 1H)	—	C-4, H(R)
3.5–3.2 (m, 4H)	simplifies	C-5 and C-2, H's(R)
[2.5	disappears	benzylic OH(Ar)§]

* In ppm relative to methyl protons of DMSO, assigned as 2.5 relative to TMS.
† Effect on spectrum following addition of deuterium oxide to the sample. Simplify indicates that the multiplicity of the splitting of the proton peaks was reduced.
‡ Ad, adenine; R, deoxyribose; Ar, aromatic.
§ Obscured by the DMSO peak.

derivative. Very little amine was formed and any dimeric products derived from the amine [12] (also only very small amounts) were completely removed by extraction into dichloromethane, as evidenced by HPLC.

After the reaction of the hydroxylamine with DNA, the DNA was hydrolysed and modified nucleosides were extracted into butan-1-ol and analysed by reversed-phase HPLC (as described in Fig. 2). Only a single UV-absorbing peak was isolated. It had the same retention time as the major adduct (adduct I) formed following the reaction of MOCA-NHOH and DNA. Using the extended water/methanol gradient (described in Fig. 5), its retention time was again identical to that of adduct I.

The large-scale reaction between *N*-hydroxy-4-amino-3-chlorobenzyl alcohol and DNA produced enough material for NMR spectroscopy. The spectrum was identical to that in Table 1, confirming that adduct I, from the reaction of MOCA-NHOH with DNA and from *N*-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA, are the same compound.

A sample of adduct I obtained from a liver slice experiment was collected on reversed-phase HPLC. This was then co-injected with the synthetic adduct on ion-exchange HPLC (Fig. 6). Under these conditions, the synthetic and radioactive adducts had identical chromatographic properties.

Characterisation of Adduct I

The NMR spectra of adduct I, formed either by reaction of MOCA-NHOH or *N*-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA, were identical and details are presented in Table 1. Aromatic protons are apparent at 7.2, 7.0 and 6.7, with splitting characteristics of 1,3,4 trisubstitution (of a single aromatic nucleus). The resonance of 6.9 is not aromatic since it is removed on addition of D_2O. The proton at 7.9 could be that of the C-8 of deoxyguanosine, or C-2 or C-8 of deoxyadenosine. The splitting of the methylene protons (4.3), which is removed on addition of D_2O, shows them to be adjacent to an exchangeable proton, either an N–H or an O–H. There is no resonance between 10 and 11 for the proton on the N-1 of deoxyguanosine, which indicates that, if the base is deoxyguanosine, it is either substituted at the N-1 position or is aromatised and substituted at the O^6 position. (Analysis of spectra were done to 15.) Since the methylene pro-

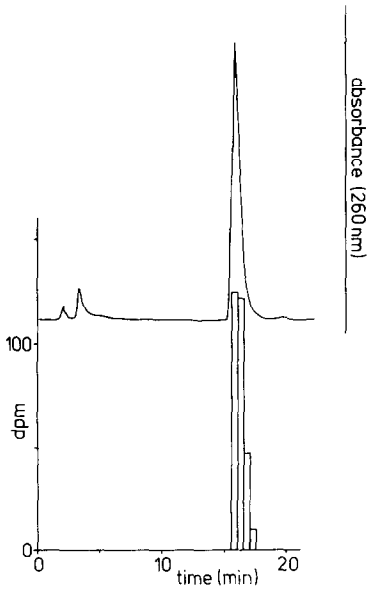

Fig. 6. Ion exchange HPLC profile of adduct I obtained following incubation of [^{3}H]MOCA with rat liver slices and previously purified by reversed phase HPLC (vertical bars) and from the reaction of *N*-hydroxy-4-amino-3-chlorobenzyl alcohol with DNA (UV trace). Ion exchange chromatography was performed using a Partisil 10 SCX column (250 mm × 4.6 mm) in the ammonium ion form, and eluted with a gradient of ammonium formate (pH 4, in 10% (v/v) methanol); 2 mM for 5 min, 2 mM–20 mM over 15 min. Flow rate was 1 ml/min, 0.5 min fractions were collected and assayed for radioactivity.

tons are split, then substitution on the nucleoside can only be via the amino group of the aromatic ring, which itself is a benzyl alcohol derivative. From the above the only possible positions of substitution consistent with this spectrum are N-1 or O^6 of deoxyguanosine, or C-8 of deoxyadenosine or C-2 of deoxyadenosine. The last possibility, however, is extremely unlikely based on known interactions of chemicals with DNA and the low reactivity of this position with electrophilic species.

Mass spectra were performed on the silylated adduct by electron impact. The spectrum showed ions at m/e 466, 394 and 376/378. The former ions, which contained a chlorine atom, were consistent with the O^6-derivative of deoxyguanosine, namely *N*-(deoxyguanosin-O^6-yl)-4-amino-3-chlorobenzyl alcohol and could represent the tri- and di-silylated adduct with loss of the aromatic ring. It remains unclear, however, how the ions at 376/378 could be formed.

Fast atom bombardment (FAB) mass spectroscopy of adduct I failed to produce a spectrum of any value. Somewhat surprisingly, however, FAB mass spectroscopy of the silylated derivative produced a spectrum which gave the mass ion with some fragmentation. In detail, major ions were observed at m/e 623 and 645 corresponding to the trisilylated mass ion plus a proton $[M^+ + H]^+$ and plus a sodium atom $[M + Na]^+$ respectively. The presence of peaks at 625 and 647 confirmed the presence of a chlorine atom. A minor peak was observed at m/e 661 corresponding to $[M + K]^+$. A minor peak was also observed at m/e 551 corresponding to the disilylated mass ion plus a proton and major ions were present at m/e 363 and 291. These latter two ions were observed to contain chlorine and correspond to monosilylated and non-silylated derivatives (respectively), which have lost the bisilylated deoxyribose moiety and two hydrogen atoms. The only other ions observed were at m/e 570, 498, 426 and 354. Clearly, these are all related, differing in 72 mass units corresponding to sequential loss of TMS groups. These ions did not contain chlorine atoms. Whilst it is not certain as to their origin, it is possible that they could derive from silylated derivatives of the mass ion which has lost both the chlorine atom and an hydroxyl group $[M–Cl–OH]^+$. Thus, the only structure consistent with both the NMR spectrum and the FAB mass spectrum is *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (Fig. 7). If the adduct were *N*-(deoxyguanosin-8-yl)-4-amino-3-chlorobenzyl alcohol, the mass ion of the trisilylated derivative should have been observed at m/e 639. No peak was present in this region.

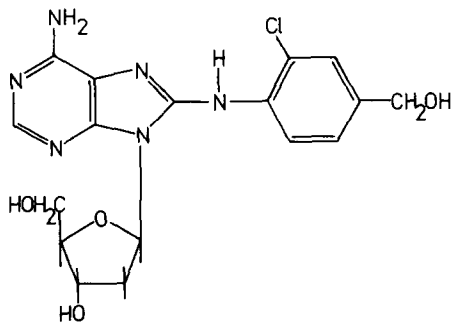

Fig. 7. Molecular structure of *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (adduct I).

DISCUSSION

The results presented show that both MOCA and AcMOCA metabolites bind to rat liver DNA following i.p. injection or following incubation with rat liver slices. Following i.p. injection of [ring-^{3}H]AcMOCA or [ring-^{3}H]MOCA, three radiolabelled adducts were detected on HPLC. The earliest eluting peak (adduct I) was not observed by scintillation counting following i.p. injection of [acetyl-^{3}H]AcMOCA, which indicates that adduct I is non-acetylated. It was not possible, however, to perform pH partitioning experiments [18] on any of the adducts, due to the low extent of DNA binding.

Reversed-phase HPLC of adducts obtained following i.p. injection of [ring-^{3}H]MOCA into rats (Fig. 2a) and adducts obtained following incubation of [ring-^{3}H]MOCA with liver slices (Fig. 3), demonstrated identical chromatographic properties of three adducts. It was found, however, that adduct I was the main adduct obtained in each case. The 25 min peak was more evident in hydrolysed DNA from *in vivo* experiments than from liver slices. In Fig. 4, the radiochromatograph from the incubation of [ring-^{3}H]MOCA with liver slices, shows none of the 25 min peak. This was unusual, the peak normally being present as a minor product (cf. Fig. 3) and may suggest it is unstable under the *in vitro* conditions used.

In order to determine the structure of the major *in vivo* adduct produced by MOCA in liver DNA, it was necessary to synthesise the presumed reactive intermediate MOCA-NHOH. The synthetic route to MOCA-NHOH was unusual when compared to the synthesis of other hydroxylamines. In cases such as the hydroxylamine derivatives of aminofluorene and the naphthylamines, there is only one amino group present enabling oxidation to the nitro function, and subsequent reduction [19]. In the case of benzidine, two amino groups are present and to synthesise 4-amino-4′-nitrobiphenyl, benzidine is oxidised to the dinitro derivative, acetylated and then selectively reduced [7]. However, attempts to selectively oxidise one amino group of MOCA using 3-chloroperbenzoic acid were unsuccessful. (Subsequent to the preparation of this manuscript, it has been learnt that this can be achieved; Dr. F. F. Kadlubar, personal communication.) The method used for the synthesis of MOCA-NO_2 was a Friedel–Crafts alkylation; 2-chloroacetanilide had to be used (rather than 2-chloroaniline) to avoid complex formation with the aluminium trichloride catalyst and, since it is a solid, a solvent (nitromethane) was also required [20]. *N*-acetylated MOCA-NO_2 so formed was hydrolysed to give MOCA-NO_2. At all stages of the reaction the product was monitored by HPLC and usually only a single UV absorbing peak of MOCA-NO_2 was observed. Any contaminating 3-chloro-4-nitrobenzyl chloride present was removed. Although it is possible that some 3-chloro-4-nitrobenzyl alcohol could have been formed from acid hydrolysis of 3-chloro-4-nitro-

benzyl chloride, none was ever seen by HPLC. However, it is possible that small amounts of 3-chloro-4-nitrobenzyl alcohol, not detected by HPLC, were present which could then react with DNA. In order to remove this possibility, two methods were used. Firstly, a sample of MOCA-NO_2 was passed down silica. With the eluent used, MOCA-NO_2 is eluted much earlier than 3-chloro-4-nitrobenzyl alcohol and so any of the latter which might have been present would have been removed. The MOCA-NO_2 was then reduced to the hydroxylamine MOCA-NHOH which was purified on HPLC and then reacted with DNA. It is inconceivable that any *N*-hydroxy-4-amino-3-chlorobenzyl alcohol was present; its elution time on the HPLC system used was some 20 min earlier than that of MOCA-NHOH. The reacted DNA was enzymically hydrolysed. HPLC analysis of the hydrolysates were identical to the UV trace shown in Fig. 4. Thus, synthetic MOCA-NHOH reacts with DNA to give adducts I and II.

The single ring hydroxylamine *N*-hydroxy-4-amino-3-chlorobenzyl alcohol was synthesised and reacted with DNA. Following enzymic hydrolysis of the DNA, only a single UV-absorbing peak was detected on reversed-phase HPLC. This had the same retention time as the synthetic adduct I formed from the reaction of MOCA-NHOH with DNA. The NMR spectrum of the synthetic adduct I from MOCA-NHOH and DNA was identical to that of the compound formed from the single ring hydroxylamine and DNA. Thus both MOCA-NHOH and *N*-hydroxy-4-amino-3-chlorobenzyl alcohol react with DNA to form adduct I.

Co-chromatography of adduct I formed from the incubation of [ring-^{3}H]MOCA with rat liver slices and synthetic adduct I was carried out. In two different gradients on reversed-phase HPLC and on ion-exchange HPLC, the chromatographic properties of the two adducts were identical, showing that the synthetic and *in vivo* adducts are the same compound.

The structure of adduct I was determined by NMR and mass spectroscopy. The NMR spectrum showed a single aromatic ring, a benzyl alcohol, attached to a deoxynucleoside, either deoxyguanosine substituted at the O^6 or N-1 positions, or deoxyadenosine at the C-8 or C-2 positions. The last possibility is extremely unlikely. Fast atom bombardment mass spectra included the mass ion of the postulated silylated deoxyadenosine adduct. Thus the only structure consistent with these data is *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (Fig. 7).

Approximately similar amounts of adduct I were formed from approximately similar amounts of the two hydroxylamines (i.e. MOCA-NHOH and *N*-hydroxy-4-amino-3-chlorobenzyl alcohol) when reacted with DNA. This suggests that MOCA-NHOH itself reacts with DNA. If breakdown to *N*-hydroxy-4-amino-3-chlorobenzyl alcohol were a prerequisite for DNA reactivity, then this latter compound would be expected to be more reactive than MOCA-NHOH. As loss of an aromatic ring has occurred, the reaction must involve cleavage of the methylene bridge. The chemical mechanism for this is unknown and further data will be required before one could be postulated.

The identification of a single ring adduct lends further weight to the argument that aromatic amines must be individually investigated as to their activation, metabolism and DNA binding characteristics. Clearly the apparent structural similarity to benzidine does not manifest itself in the DNA adducts produced. The methylene bridge in MOCA, as well as altering the orientation of the two aromatic rings (i.e. at two corners of a tetrahedron for MOCA, as opposed to being at 180° for benzidine), also prevents delocalisation of electrons between the two aromatic rings. This may explain why at least one single ring adduct is formed. Results obtained in the *Salmonella* reversion assay [21], where the mutagenicity of MOCA is greater than that of AcMOCA are consistent with the fact that, unlike benzidine, *N*-acetylation is not required for DNA binding. Similarly, the rate of acetylation of rabbit hepatocytes was found not to affect the rate of DNA damage caused by MOCA [22].

Thus, following i.p. injection of [ring-^{3}H]MOCA, three DNA adducts are detected. While two remain unidentified, the structure of the earliest eluting peak on HPLC appears to be *N*-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol, a compound involving a single aromatic nucleus attached to a deoxyadenosine residue via the C8 position.

Acknowledgements—We would like to thank Drs. I. H. Sadler, D. Reed, A. J. G. Crawshaw and J. R. Lindsay-Smith for the performing of, and help with the interpretation of, NMR spectra, Dr. T. Dransfield for obtaining some of the mass spectra and Dr. D. P. Cooper and Dr. F. F. Kadlubar for helpful discussions. We are grateful to Mrs. Jenny Duckmanton for the careful preparation of this manuscript.

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