Macromolecular Binding and Metabolism of the Carcinogen 4-Chloro-2-methylaniline

Donald L. Hill, Tzu-Wen Shih, and Robert F. Struck

Kettering-Meyer Laboratory, Southern Research Institute, Birmingham, Alabama 35205

ABSTRACT

Biochemical investigations relating to the mechanism of action and mechanism of activation have been made for the carcinogen, 4-chloro-2-methylaniline. Radioactivity from 4-chloro-2-\([\text{methyl}^{14}\text{C}]\)methylaniline became extensively bound to protein, DNA, and RNA of rat liver, but macromolecules of some of the other tissues examined contained little radioactivity.

Enzymatic activity dependent upon reduced nicotinamide adenine dinucleotide and leading to irreversible binding of radioactivity from labeled 4-chloro-2-methylaniline to macromolecules in the reaction system was present in microsomes from rat liver. The activity was inducible by phenobarbital. Two soluble products of microsomal enzymes were identified by mass spectral analysis and chemical synthesis as 5-chloro-2-hydroxylaminotoluene and 4,4'-dichloro-2,2'-dimethyloxobenzene. The hydroxylamino compound appears to be a more activated form of 4-chloro-2-methylaniline.

INTRODUCTION

4-Chloro-2-methylaniline, a metabolite of chloroform, a widely used acaricide (13), is one of many potentially carcinogenic compounds to which humans are exposed. When administered in the diet of rats, it causes hemangiosarcomas in various organs (18). The purpose of the present study was to investigate biological mechanisms by which 4-chloro-2-methylaniline is activated and by which it might act as a carcinogen.

MATERIALS AND METHODS

Preparation of Labeled 4-Chloro-2-methylaniline. 4-Chloro-2-\([\text{methyl}^{14}\text{C}]\)methylaniline hydrochloride was prepared by the nitration of methyl-labeled toluene followed by reduction with stannous chloride in hydrochloric acid (9). The chemical purity of the preparation was 97%, and no radioactive impurities were evident. The specific activity was 3.0 mCi/mmol.

In Vivo "Binding" to Macromolecules. Five Osborne-Mendel rats weighing between 340 and 387 g were given i.p. injections of 4-chloro-2-\([\text{methyl}^{14}\text{C}]\)methylaniline hydrochloride at a dose of 14 mg/kg (81 to 93 \(\mu\)Ci) in a 0.9% NaCl solution. At 24 hr after treatment, the rats were killed by asphyxiation in CO\(_2\) and dissected. Tissues were collected and pooled according to type.

Macromolecules were extracted by a modification of the Kirby procedure (6, 12). The tissues were homogenized in 5 volumes of a 6% solution of the sodium salt of \(p\)-aminosalicylic acid with a tight-fitting Potter-Elvehjem glass homogenizer. A 10% solution of sodium dodecyl sulfate (0.5 ml) was added. The resulting viscous solution was extracted with an equal volume of a solution of phenol:m-chloro:8-hydroxyquinoline:water (500:70:0.5:55, by weight) and centrifuged at 11,000 \(\times\) g for 30 min. DNA was precipitated from the aqueous layer by the addition of an equal volume of cold 2-ethoxyethanol. The precipitate was collected by centrifugation, and 2 volumes of cold ethanol were added to the remaining aqueous phase to precipitate RNA. The phenol layer was extracted once again with the solution of \(p\)-aminosalicylate and added to an excess of methanol to precipitate proteins. The DNA was dissolved in 2 ml of 1 mM potassium phosphate (pH 7.0), incubated at 37\(^\circ\)C for 15 min with RNase (50 \(\mu\)g/ml), extracted again with the phenol reagent, and precipitated as before with 2-ethoxyethanol. The DNA and RNA were washed successively with ethanol and ether. The protein fraction was washed 4 times with methanol, once with acetone, and finally with ether. The concentration of nucleic acids, dissolved in 1 mM potassium phosphate (pH 7.0), was calculated from the absorbance at 260 nm, with the assumption that at this wavelength \(E_{1\text{cm}^1}\text{ml} = 200\) for DNA and 250 for RNA. Protein was measured by the method of Lowry et al. (14). Prior to scintillation counting, a portion of the DNA, RNA, or protein was dissolved in 1 ml of Soluene 350 (Packard Instrument Co., Downer's Grove, Ill.), and toluene:Liquiflour scintillator was added.

Microsomal Binding Reaction. For these reactions, enzymatic activity was induced by giving rats i.p. injections of phenobarbital (100 mg/kg) consecutively for 2 days. Microsomes were prepared as described previously (5). The incubation mixture contained 20 \(\mu\)mol of Tris-chloride (pH 8.0), 300 nmol of NADPH, 56 nmol of labeled 4-chloro-2-methylaniline hydrochloride, and washed microsomes equivalent to 50 mg of liver, wet weight, in a total of 500 \(\mu\)l. Incubation was at 37\(^\circ\)C. Each assay was performed in duplicate and had duplicate controls lacking NADPH. Acetone (500 \(\mu\)l) was added to stop the reaction. The preparations were extracted twice with 1 ml of chloroform:meanol (2:1). To the remaining aqueous solution, 1 ml of 10% trichloroacetic acid was added. The precipitate was collected by centrifugation, washed with 5% trichloroacetic acid and with acetone, and collected on Whatman GF/A glass fiber filters. The radioactivity on the filter was determined by scintillation counting after digestion in 1 ml of Soluene 350.

Microsomal Oxidation to Soluble Metabolites. The standard reaction system contained, in a total of 200 \(\mu\)l, 0.22 \(\mu\)mol of labeled 4-chloro-2-methylaniline hydrochloride, 12 \(\mu\)mol of Tris chloride buffer (pH 7.4), 0.4 \(\mu\)mol of NADPH, and 20 \(\mu\)l of washed microsomes (equivalent to 20 mg of liver, wet weight) from livers of phenobarbital-induced rats. The reactions were
Metabolism of 4-Chloro-2-methylaniline

stopped after 10 min by immersion in a boiling water bath for 1 min. A portion (50 μl) of each preparation was streaked onto a paper strip for chromatography in a solvent of 0.3 M NaCl. Easy assay was prepared in duplicate, and each had duplicate controls lacking NADPH. When the strips were dried, unchanged 4-chloro-2-methylaniline was sublimed from the strips. (The total radioactivity added was calculated by developing the strips in 0.3 M H3PO4; no sublimation of substrate occurred under these conditions, but the metabolite peak was obscured by the substrate.)

Synthesis of 4,4'-Dichloro-2,2-dimethylazobenzene and 5-Chloro-2-hydroxylaminotoluene. 4,4'-Dichloro-2,2-dimethylazobenzene was prepared from 4-chloro-2-methylaniline by oxidation with hydrogen peroxide (Ref. 4, p. 531). 4-Chloro-2-methylaniline (200 mg) in 1 ml ethanol, 1 ml of water, and 1 ml of 30% hydrogen peroxide was refluxed for 18 hr with stirring. The dark mixture was evaporated to dryness in a vacuum, and the residue in methanol was separated by preparative TLC3 on silica gel in acetone:chloroform (1:3). The top, visible band was eluted with methanol to isolate the crude product in 40% yield. Purification of the crude product was accomplished by crystallization from cyclohexane, giving orange needles; m.p. 161°; IR (cm⁻¹): 2950, 2915, 1585, 1565, 1465, 1435, 1390, 1370, 1290, 1210, 1185 (s), 1110, 1085, 885, 865, 815 (s), 655, 585, 560, 465, 375, 360; UV (nm) in ethanol: 237 (sh), 241, 247 (sh), 344; mass spectrum (m/e): 278 (2 Cl), M⁺, 263 (2 Cl, M — CH₃), 241 (2 Cl, M — Cl), 153 (1 Cl, M — (CH₃)₂). (Chart 1).

N=N

(6 H, singlet, CH₃), 7.13 to 7.24 (2 H, doublet of doublets, H para to CH₃), 7.29 to 7.31 (2 H, doublet, H ortho to CH₃), 7.50 to 7.60 (2 H, doublet, H ortho to N); carbon, hydrogen and nitrogen elemental analysis satisfactory for C₇H₆CINO₂. These data establish the structure of this product as 4,4'-dichloro-2,2'-dimethylazobenzene.

4-Chloro-2-methylaniline was converted to 5-chloro-2-nitrotoluene by a reported procedure (16). 4-Chloro-2-methylaniline (14.1 g) in 125 ml of 4 n HCl (incomplete solution) in an ice bath was treated dropwise in 15 min with 6.9 g of NaN₂ in 25 ml of water. The mixture was stirred for 1 hr in the ice bath and treated in one batch with 17 g of NaBF₄ in 50 ml of water. Filtration gave a white solid, which was washed with 10 ml of cold water, ethanol, and ether and dried in a vacuum: yield, 13.2 g of 4-chloro-2-methylphenyldiazonium tetrafluoroborate. The solid (13.2 g) suspended in 50 ml of water was added batchwise in 10 min to a vigorously stirred solution of 44 g of NaN₂ in 100 ml of water containing 8.8 g of copper powder. The mixture was stirred for 10 min and allowed to settle, and the solution was decanted from the copper and extracted with chloroform. The extract was dried over NaN₂, filtered, and evaporated in a vacuum, giving a dark oil. The oil was distilled at high vacuum (0.1 mm) through a short-path distillation apparatus and gave a clear, colorless distillate; m.p. 24° (reported m.p. 24°) (3); IR (cm⁻¹): 3100, 2980, 2930, 2850, 1600, 1570, 1515, 1465, 1445, 1380, 1340, 1295, 1200, 1150, 1105, 1075, 1030, 890, 825, 800, 750, 730, 675, 545, 480, 420; UV (nm) at pH 7: 218 (sh), 273; mass spectrum (m/e): 171 (1 Cl, M⁺), 154 (1 Cl, M — OH⁻), 126 (1 Cl, M — (NO) — (CH₂)₄), 125 (1 Cl, M — NO₂); PMR (6) (after addition of shift reagent Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanediene)europium III [Eu(FOD)]: 2.70 (s, CH₃) 7.32 to 7.42 (doublet, H ortho to Cl and para to CH₃), 7.40 (s, H ortho to CH₃), 8.08 to 8.18 (doublet, H ortho to NO₂); carbon, hydrogen and nitrogen elemental analysis satisfactory for C₇H₆ClNO₂. These data establish the structure of this product as the previously reported 5-chloro-2-nitrotoluene (3).

5-Chloro-2-nitrotoluene was converted to 5-chloro-2-hydroxylaminotoluene by a reported procedure (17). The nitro derivative (400 mg) suspended in 20 ml of ethanol:water (3:1) was treated with 450 mg of NH₄Cl. Zinc dust (95%, 780 mg) was added batchwise in 10 mm to a vigorously stirred mixture. After stirring 15 min at room temperature, the mixture was filtered through celite. Evaporation of ethanol from the filtrate in a vacuum gave a mixture, which was extracted with 10 ml of chloroform. The chloroform extract was dried over Na₂SO₄, filtered, and evaporated to dryness in a vacuum. The product was then treated in one batch with 17 g of NaNO₂ in 50 ml of water containing 8.8 g of copper powder. The mixture was refluxed for 18 hr with stirring. Paper chromatography in a solvent of 0.3 M NaCl. The extract was treated dropwise in 15 mm with 6.9 g of NaNO₂ in 25 ml of water containing 8.8 g of copper powder. The mixture was stirred for 10 min and allowed to settle, and the solution was decanted from the copper and extracted with chloroform. The extract was dried over NaN₂, filtered, and evaporated in a vacuum, giving a dark oil. The oil was distilled at high vacuum (0.1 mm) through a short-path distillation apparatus and gave a clear, colorless distillate; m.p. 24° (reported m.p. 24°) (3); IR (cm⁻¹): 3100, 2980, 2930, 2850, 1600, 1570, 1515, 1465, 1445, 1380, 1340, 1295, 1200, 1150, 1105, 1075, 1030, 890, 825, 800, 750, 730, 675, 545, 480, 420; UV (nm) at pH 7: 218 (sh), 273; mass spectrum (m/e): 171 (1 Cl, M⁺), 154 (1 Cl, M — OH⁻), 126 (1 Cl, M — (NO) — (CH₂)₄), 125 (1 Cl, M — NO₂); PMR (6) (after addition of shift reagent Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanediene)europium III [Eu(FOD)]: 2.70 (s, CH₃) 7.32 to 7.42 (doublet, H ortho to Cl and para to CH₃), 7.40 (s, H ortho to CH₃), 8.08 to 8.18 (doublet, H ortho to NO₂); carbon, hydrogen and nitrogen elemental analysis satisfactory for C₇H₆ClNO₂. These data establish the structure of this product as the previously reported 5-chloro-2-nitrotoluene (3).

RESULTS

In Vivo Binding to Macromolecules. The results of studies on the binding of radioactivity from labeled 4-chloro-2-methylaniline to macromolecules of various organs are shown in Table 1. The liver of rats contained the largest amounts of radioactivity bound to protein, DNA, and RNA. Macromolecules of brain, heart, testis, and muscle contained little bound radioactivity. In a study of the tissue distribution of radioactivity from 4-chloro-2-[methyl-¹⁴C]methylaniline (not shown), we observed that at 24 hr after an i.p. dose the livers of rats contained more radioactivity than did the combined amounts in all of the other organs listed in Table 1.

In Vitro Binding to Macromolecules. In mat liver microsomes, enzymatic activity leading to the irreversible binding of radioactivity from labeled 4-chloro-2-methylaniline was present (Chart 1). The activity was dependent upon the presence of NADPH and was increased by pretreatment of the rats with phenobarbital.

Microsomal Oxidation to Soluble Metabolites. Paper chromatography of 4-chloro-2-methylaniline and its metabolites...
formed by microsomal enzymes from livers of rats pretreated with phenobarbital revealed a metabolite peak remaining at the origin. Optimum conditions for the formation of the material in this peak were established (Chart 2). The reaction proceeded in a linear fashion for 10 min, and the rate of reaction was proportional to the amount of microsomes added. Above a concentration of 1 mM, NADPH, which was required, did not greatly influence the reaction rate. The optimum pH was 7.4, and the rate of reaction increased with substrate concentration in the range of 0.1 and 1.2 mM. Under optimum conditions, 50 nmol of products were formed in this assay. No reaction was detectable in rats not pretreated with phenobarbital.

A large-scale preparation of soluble microsomal metabolites was subjected to paper chromatography in 0.3 M NaCl, and the material remaining at the origin was eluted with ethanol. Preparative TLC of the eluted material in heptane gave a major radioactive band at the origin and another at RF 0.6. Elution of the mobile component with methanol and mass spectral analysis tentatively identified it as 4,4'-dichoro-2,2'-dimethylazo-benzene. The structure was confirmed by synthesis of the compound, which had a mass spectrum identical to that of the metabolite. The major radioactive band at the origin upon TLC in heptane was found to be identical to the major metabolite in a chloroform extract of the reaction mixture (see below).

Extraction of the reaction mixture with chloroform, with or without prior adjustment to pH 12, revealed that no detectable metabolites remained in the aqueous phase; no products were observed on paper chromatography or TLC. This eliminated the possibility of the presence of substantial amounts of phenolic metabolites in this system and indicated that side-chain hydroxylation had occurred. In addition, treatment of a chloroform extract of the reaction mixture with diazomethane failed to alter the TLC migration of the main or minor metabolite.

The major metabolite was purified by extracting a large-scale reaction mixture with chloroform and sequential TLC in chloroform (RF 0.05) and twice in chloroform:methanol (97:3) (RF 0.45). It was eluted from the plates with methanol. The mass spectrum of the metabolite was identical to synthetic 5-chloro-2-hydroxylaminotoluene. Further confirmation of the identity of the major metabolite was obtained by cochromatography of the metabolite and synthetic 5-chloro-2-hydroxylaminotoluene by TLC in several solvent systems, as shown in Table 2.

### DISCUSSION

Results of our studies with intact rats show that radioactivity from 4-chloro-2-[methyl-14C]methylaniline becomes bound to macromolecules in tissues. Liver contains the largest amount of radioactivity, both bound and total. An enzymatic process catalyzed by liver microsomes of rats and leading to irreversible binding may be involved in the in vivo binding, and such a

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Irreversible binding of radioactivity from 4-chloro-2-[methyl-14C]-methyl aniline to protein, DNA, and RNA of tissues of rats given i.p. injections of this agent 24 hr previously</th>
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<tr>
<td>Tissue</td>
<td>Protein</td>
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<th>Table 2</th>
<th>Thin-layer cochromatography of synthetic 5-chloro-2-hydroxylaminotoluene and the major microsomal metabolite of 4-chloro-2-methylaniline</th>
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<td>Solvent</td>
<td>RF</td>
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<td>Benzene:ethyl acetate (1:1)</td>
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<tr>
<td>Ether:heptane (3:7)</td>
<td>0.15</td>
</tr>
<tr>
<td>Chloroform:methanol (95:5)</td>
<td>0.42</td>
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<tr>
<td>Chloroform:methanol (3:1)</td>
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<td>Acetone:chloroform (1:9)</td>
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<td>1,2-Dimethoxyethane</td>
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<td>Acetonitrile:1,2-dimethoxyethane (1:1)</td>
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<tr>
<td>Ether</td>
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<tr>
<td>Cyclohexane:1,2-dimethoxyethane (1:1)</td>
<td>0.95</td>
</tr>
<tr>
<td>Acetonitrile:acetone:benzene:cyclohexane (4:5:2:5)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Chart 1.** 4-Chloro-2-[methyl-14C]methylaniline as a substrate for microsomal enzymes that catalyze a reaction leading to irreversible binding of the substrate to microsomal enzymes. ○, no pretreatment, NADPH omitted; △, phenobarbital pretreatment, NADPH omitted; ●, no pretreatment, NADPH present; Δ, phenobarbital pretreatment, NADPH present. Ordinate, nmol of bound product per assay.

**Chart 2.** Production of soluble metabolites of 4-chloro-2-[methyl-14C]methylaniline (CME). A, dependence of reaction rate on amount of microsomes added; B, dependence of reaction rate on time; C, effect of NADPH; D, pH optimum (○, Tris-Cl buffer; △, phosphate buffer); E, effect of substrate. Ordinates, arbitrary units derived from the radiochromatogram scanner.
reaction may be related to the carcinogenic activity of this compound.

In the microsomal binding reaction, a reactive intermediate is apparently formed, and this product binds to groups on the microsomal proteins. This type of reaction, leading to irreversible binding of radioactivity from 4-chloro-2-{methyl-¹⁴C}methylaniline, is similar to that for the binding of vinyl chloride (10), 1,2-dibromoethane (6), and benzo(a)pyrene (5). The fact that the activity increases in the livers of rats pretreated with phenobarbital implies that cytochrome P-450 is involved in this reaction. If such binding is related to the biological activity of 4-chloro-2-methylaniline, pretreatment of rats with phenobarbital might enhance its effect.

At this time, we do not know if 5-chloro-2-hydroxylaminotoluene, a microsomal metabolite, is responsible for the irreversible binding. This N-hydroxy derivative may require, for activation, further oxidation to the nitroso derivative. Arylhdroxylamines commonly undergo dismutation and autoxidation in neutral solutions to give the corresponding nitroso derivative. The nitroso moiety is very reactive, entering into many reactions characteristic of aldehydes and ketones (4).

Acetylation or sulfation of the hydroxylamine derivative may occur in intact animals (15). That the nitroso derivative is formed is implied by the likely reaction sequence leading to the azo compound (Chart 3).

Earlier work by others on the metabolism of 4-chloro-2-methylaniline led to chromato graphic identification of 2 minor urinary metabolites, 4-chloro-2-methylacetanilide and 5-chloroantranilic acid (13). These products would be formed by acetylation of 4-chloro-2-methylaniline and oxidation of the methyl group, respectively. It is not likely that acetylation would occur in our in vitro tests. Oxidation of the methyl group, however, should have been noted if it occurred to an appreciable extent. Since 5-chloro-2-hydroxylaminotoluene is unstable, one would not expect it to appear in the urine. Acetylation of the parent compound and oxidation, even to a small extent, of the methyl group could give rise, respectively, to 4-chloro-2-methylacetanilide and 5-chloroantranilic acid as urinary metabolites.

The N-hydroxy derivative of p-chloroacetanilide has been isolated from reaction systems containing microsomes from hamster liver (7). This reaction, however, is stimulated by prior treatment of the animals with 3-methylcholanthrene and not by phenobarbital. The difference in inducibility may be due to a difference in enzymes for the 2 species, or the presence of the acetyl group may make p-chloroacetanilide a substrate for an oxidase distinct from that hydroxy lating free amines.

Microsomal enzymes are known to catalyze the oxidation of aniline to phenylhydroxylamine (8); and peroxidase and aniline oxidase of fungi oxidize aniline and its derivatives to azo compounds, presumably through the hydroxylamine and nitroso derivatives (1, 11). 4-Chloro-2-methylaniline is a substrate for these enzymes (1, 2), and its corresponding azo derivative has been identified by gas chromatography. Our data show that such azo compounds can be formed in enzyme systems from mammalian cells.

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REFERENCES

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