The metabolic activation of the environmental carcinogen 5-methylchrysene was studied by combining high-pressure liquid chromatographic analysis of metabolites formed in vitro with assays of these metabolites for mutagenic activity toward Salmonella typhimurium. Metabolites were formed by incubation of 5-methylchrysene with the 9000 x g supernatant from Aroclor-treated rat livers. With the use of reverse-phase columns, the metabolites were resolved into nine peaks, A to I. Each peak was collected and tested for mutagenicity with activation. Significant mutagenic activity was observed primarily in Peak E and to a lesser extent in Peak D. None of the other metabolites showed significant mutagenic activity. The major mutagenic metabolite (Peak E) was identified as 1,2-dihydro-1,2-dihydroxy-5-methylchrysene (7.0% from 5-methylchrysene); Peak D was 7,8-dihydro-7,8-dihydroxy-5-methylchrysene (2.5% from 5-methylchrysene). Other metabolites included 9,10-dihydro-9,10-dihydroxy-5-methylchrysene, 9-hydroxy-5-methylchrysene, 7-hydroxy-5-methylchrysene, 1-hydroxy-5-methylchrysene, and 5-hydroxymethylchrysene. These results indicate that 1,2-dihydro-1,2-dihydroxy-5-methylchrysene is a major proximate mutagen of 5-methylchrysene.

ABSTRACT

The environmental carcinogen, 5-MeC3 (Chart 1), is more carcinogenic on mouse skin and more mutagenic toward S. typhimurium than are the other methylchrysenes isomers and chrysene (2-4). The carcinogenic activity of 5-MeC, which occurs in tobacco smoke, is equivalent to that of benzo(a)pyrene whereas the other methylchrysenes isomers and chrysene are at most only weakly carcinogenic (4, 6). In view of the importance of the methylchrysene as environmental agents, the metabolic activation of 5-MeC has been studied to elucidate the mechanisms of action and the structural requirements for carcinogenicity among the methylchrysene and other methylated polynuclear aromatic hydrocarbons.

Recent mouse skin bioassays and mutagenicity assays of a series of fluorinated 5-MeC derivatives indicated that positions 1, 3, and 12 of 5-MeC (see Chart 1) are important in its metabolic activation, whereas positions 6, 7, 9, and 11 are not involved to any significant extent (5, 7). For determination of the nature of the proximate and/or ultimate active metabolites of 5-MeC formed in vitro, high-pressure liquid chromatographic analysis has now been used in conjunction with mutagenicity assays of these metabolites in S. typhimurium TA 100. The results presented here demonstrate that 5-MeC-1,2-diol is a proximate mutagen of 5-MeC and support the hypothesis that carcinogenic polynuclear aromatic hydrocarbons can be activated through formation of specific angular ring diol-epoxides (8).

MATERIALS AND METHODS

Apparatus. High-pressure liquid chromatography was done with a Waters Associates Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, a Model 660 solvent programmer, a Model 16K septumless injector, a Model 440 UV/visible detector, and Columns 1 (3.9 mm x 30 cm µBondapak/C8; Waters, Inc., Milford, Mass.) and 2 (9.4 mm x 50 cm Whatman Magnum 9 ODS; Whatman, Inc., Clifton, N. J.). Mass spectrometry was done with a Hewlett-Packard Model 5982A instrument. UV spectra were run on a Cary Model 118 spectrometer in methanol or methanol-H2O. Liquid scintillation counting was done with a Nuclear-Chicago Isocap 300 scintillation system.

Chemicals. All solvents were spectroquality. NADP+ and glucose 6-phosphate were obtained from Sigma Chemical Co., St. Louis, Mo. TCPO was obtained from Aldrich Chemical Co., Milwaukee, Wis. Aroclor-1254 was obtained from Analabs, Inc., Hamden, Conn. 5-MeC and [5-14C]-5-MeC were synthesized by published procedures and were greater than 99% pure according to analysis by high-pressure liquid chromatography and gas-liquid chromatography (4, 12). The reference compounds, 1-OH-5-MeC, 2-OH-5-MeC, 3-OH-5-MeC, 7-OH-5-MeC, 8-OH-5-MeC, 9-OH-5-MeC, 12-hydroxy-5-methylchrysene, and 5-hydroxymethylchrysene were synthesized by unambiguous methods, which will be described separately.4

1 This study was supported by Grant CA-012376 from the National Cancer Institute. This is Paper 9 of the series, "A Study of Chemical Carcinogenesis."
2 Recipient of National Cancer Institute Research Career Development Award 5K04 CA-00124. To whom requests for reprints should be addressed.
3 The abbreviations used are: 5-MeC, 5-methylchrysene; 5-MeC-1,2-diol, 1,2-dihydro-1,2-dihydroxy-5-methylchrysene; TCPO, 1,1,1-trichloropropene oxides; 1-OH-5-MeC, 1-hydroxy-5-methylchrysene; 2-OH-5-MeC, 2-hydroxy-5-methylchrysene; 3-OH-5-MeC, 3-hydroxy-5-methylchrysene; 7-OH-5-MeC, 7-hydroxy-5-methylchrysene; 8-OH-5-MeC, 8-hydroxy-5-methylchrysene; 9-OH-5-MeC, 9-hydroxy-5-methylchrysene; DMG0, dimethyl sulfoxide; 5-MeC-7,8-diol, 7,8-dihydro-7,8-dihydroxy-5-methylchrysene; chrysene-1,2-diol, 1,2-dihydro-1,2-dihydroxychrysene.

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1,2-Dihydro-1,2-dihydroxy-5-methylchrysene, a Major Activated Metabolite of the Environmental Carcinogen 5-Methylchrysene1
Metabolism of 5-MeC. For metabolic experiments in vitro, the 9000 x g supernatant was prepared from the livers of male F-344 Aroclor-treated rats (250–300 g), as previously described (10). For analysis of metabolites, a solution containing 16.3 mg MgCl₂, 24.6 mg KCl, 14.1 mg glucose 6-phosphate, and 34.0 mg NADP⁺ in a total volume of 850 µl H₂O was prepared and diluted to 5.0 ml with Tris-HCl buffer, pH 7.5. This was added to 5.0 ml of the 9000 x g supernatant (38 mg/ml protein) to give Mixture x. Each incubation flask contained 200 µl of Mixture x and 25 µg (0.10 µmol, 0.5 x 10⁸ dpm) of [5-¹⁴C]-5-MeC in 10 µl of DMSO. For preparative scale isolation and mutagenicity assays of metabolites, each flask contained 2.0 ml of Mixture x and 250 µg of 5-MeC in 100 µl of DMSO. For incubations with TCPO 7.5 µl of a methanolic solution of TCPO were added to the incubation mixture to give a final TCPO concentration of 2 x 10⁻³ M. All incubations were done at 37° for 20 min. Reactions were stopped by the addition of 1 ml ice-cold acetone (analytical scale) or 10 ml (preparative scale) followed by twice that volume of ethyl acetate. Metabolites and unchanged 5-MeC were extracted with ethyl acetate (13% remained in the H₂O). The combined ethyl acetate layers were dried (MgSO₄), concentrated, and redissolved in methanol for analysis by high-pressure liquid chromatography with Column 1 and a methanol:H₂O gradient (initial conditions, 50% methanol:50% H₂O for 30 min to final conditions, 80% methanol:20% H₂O in 40 min) at a flow rate of 3.0 ml/min and a system pressure of 2000 to 4000 psi. Metabolites were quantified by collecting each peak and determining radioactivity. For isolation of metabolites and/or mutagenicity assays, material from 5.0 mg (0.021 mmol) 5-MeC was first chromatographed on Column 1 with a methanol:H₂O gradient as above with a flow rate of 7.0 ml/min and a system pressure of 4000 to 5000 psi. Fractions were collected and further chromatographed on 2 Columns 1, as described above. The resulting fractions were concentrated on a rotary evaporator at 25° to remove most of the methanol and then lyophilized to dryness. The flasks were washed with 10 ml methanol, and the washings were concentrated for mutagenicity assays, UV and mass spectral analysis, and dehydrations. Recovery of metabolites was 90 to 95%.

Mutagenicity of Metabolites. Mutagenicity assays were done as previously described with S. typhimurium TA 100, obtained from Dr. B. N. Ames, University of California, Berkeley (1). Enzymatic activation was done with the same 9000 x g supernatant that was used in the metabolism studies. Metabolites were dissolved in 50 µl DMSO and added to plates with 200 µl (7.6 mg protein) of the 9000 x g supernatant. Two series of assays, 1 and 2, were done. In assay 1 metabolite fractions from Column 2 were assayed. The fractions contained Peaks A, B to E, F, and G to I (see Chart 2) and all material eluting before and after each peak, such that the entire chromatogram was collected. All material from each peak was assayed. The metabolites were from 3.10 µmol of 5-MeC. In Assay 2 the fraction from Column 2 containing Peaks B to E was rechromatographed on 2 Columns 1, and all material from each of the resulting individual Peaks B to E was assayed. The metabolites were formed from 6.20 µmol of 5-MeC. In each series of assays, each plate was run in duplicate. For assays of 5-MeC a dose-response curve was constructed; maximum mutagenicity was obtained for 20 µg 5-MeC per plate.

Dehydrations of Diols. The metabolite suspected to be a diol was dissolved in 25 ml of H₂O at pH 2 containing a catalytic amount of p-toluenesulfonic acid. The solution was heated at 80° for 30 min and extracted with CHCl₃ (10 ml); the CHCl₃ was concentrated, and the residue was redissolved in methanol for analysis by high-pressure liquid chromatography on Column 1 with 65% methanol in H₂O at 2.0 ml/min. These conditions were optimal for separation of the chrysenols. The relative retention volumes were: 3-OH-5-MeC, 0.78; 8-OH-5-MeC, 0.78; 9-OH-5-MeC, 0.81; 2-OH-5-MeC, 0.82; 7-OH-5-MeC, 0.87; 1-OH-5-MeC and 12-hydroxy-5-methylchrysene, 1.00; the absolute retention volume of 1-OH-5-MeC was 70.7 ml.

RESULTS

Chart 2 shows the high-pressure liquid chromatographic metabolite profile obtained after incubation of 5-MeC with the 9000 x g supernatant from Aroclor-treated rat livers. To determine which metabolites could be proximate muta-
gens of 5-MeC, preparative scale incubations were done, and fractions were collected containing peaks as summarized in Table 1. Each fraction was assayed for mutagenicity toward S. typhimurium TA 100 with activation (Assay 1). Only the fraction including Peaks B to E showed significant mutagenic activity. The mutagenicity of this fraction was equivalent to the maximum mutagenicity obtained when 5-MeC was assayed with the use of S. typhimurium TA 100 with activation [521 ± 67 (S.D.) His + revertants/plate].

For determination of which components were responsible for the observed mutagenicity, Peaks B to E were each tested (Assay 2). The results, as shown in Table 1, indicate that Peak E was the major metabolite of 5-MeC capable of further activation to a mutagen; some activity was also observed for Peak D.

The structures of Metabolites A to I of 5-MeC are also summarized in the table. Peak A was identified as 9,10-dihydro-9,10-dihydroxy-5-methylchrysene by its mass spectrum (M+ 276); its UV spectrum, which was similar to that of 3,4-dihydro-3,4-dihydroxychrysene (9); and dehydration to 9-OH-5-MeC. The latter was identified by comparison of its UV spectrum with a reference sample and by coinjection with a reference sample under high-pressure liquid chromatographic conditions that separated the various chrysenols. Peaks D and E were similarly identified as 5-MeC-7,8-diol and 5-MeC-1,2-diol, respectively. Each compound gave an UV spectrum similar to that reported for chrysene-1,2-diol and had a molecular ion of 276. On dehydration Peak D gave a mixture of 7-OH-5-MeC (93%) and 8-OH-5-MeC (7%), while Peak E gave 1-OH-5-MeC (95%) and 2-OH-5-MeC (5%). Peaks A, D, and E disappeared when the incubations were done in the presence of TCPO, an inhibitor of epoxide hydrolase (11). The configuration of diols A, D, and E are unknown, but it is probable that they are trans-diols by analogy to previous results (8). Peak F was identified by comparison of its UV and mass spectra and chromatographic retention volume to a reference sample of 5-hydroxymethylchrysene. The UV and mass spectra and chromatographic retention volumes of Peaks G to I led to their tentative identification as 9-OH-5-MeC, 7-OH-5-MeC, and 1-OH-5-MeC. A minor component eluting prior to 9-OH-5-MeC was tentatively identified as 3-OH-5-MeC. These structural assignments are based on available standards (see "Materials and Methods") and do not exclude other chrysenols.

As shown in the table, 5-MeC-1,2-diol and 5-hydroxymethylchrysene were major metabolites isolated in these in vitro experiments. The ratio of 5-MeC-1,2-diol to 5-MeC-7,8-diol was 2.7:1. When protein concentration was decreased from one-half to one-eighth of the usual amount, there was a corresponding linear decrease in diol formation, but the ratio of 5-MeC-1,2-diol to 5-MeC-7,8-diol remained constant.

**DISCUSSION**

The results indicate that 5-MeC-1,2-diol is a proximate mutagen of 5-MeC. Among the other metabolites observed, only 5-MeC-7,8-diol showed significant mutagenic activity. Since identical aliquots of all metabolic fractions were used for mutagenicity assays, the lower mutagenicity obtained for 5-MeC-7,8-diol was probably a reflection of the quantity tested rather than of its structural features. Quantitative evaluation of the relative mutagenic activity of each diol awaits their syntheses, which are currently in progress. The results reported here are in agreement with a recent study on the mutagenicity of synthetic chrysene-1,2-diol, 3,4-dihydro-3,4-dihydroxychrysene, and 5,6-dihydro-5,6-dihydroxychrysene (13). Only chrysene-1,2-diol showed significant activity toward S. typhimurium TA 100 in the presence of hepatic microsomes, and this activity was probably due to formation of 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydrochrysene.

The involvement of 5-MeC-1,2-diol as a proximate form of 5-MeC is consistent with recently completed studies on the tumor-initiating activity of a series of fluorinated derivatives of 5-MeC (5). Thus, 1-fluoro-5-methylchrysene and 3-fluoro-5-methylchrysene, in which formation of 5-MeC-1,2-diol and 1,2-dihydroxy-3,4-epoxy-1,2,3,4-tetrahydro-5-methylchrysene would presumably be blocked, were both significantly less active as tumor initiators than was 5-MeC. On the other hand 7-fluoro-5-methylchrysene and 9-fluoro-5-methylchrysene were as active as was 5-MeC, presumably because 5-MeC-7,8-diol and the corresponding diol-epoxide were relatively minor metabolites in each case. The results of this study are in agreement with the "bay region" hypothesis of polynuclear aromatic hydrocarbon carcinogenesis and support its extension to methylated compounds (8). This theory predicts that 5-MeC-1,2-diol and 5-MeC-7,8-diol are candidates for proximate forms of the carcinogen 5-MeC. However, in the *in vitro* experiments reported here there was a distinct metabolic preference for

**Table 1**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Structure</th>
<th>nmol</th>
<th>Assay 1</th>
<th>Assay 2</th>
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<tr>
<td>A</td>
<td>5-MeC-9,10-diol</td>
<td>5.7</td>
<td>110 ± 11</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Unknown</td>
<td>0.37</td>
<td>110 ± 11</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Unknown</td>
<td>1.4</td>
<td>562 ± 12</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>D</td>
<td>5-MeC-7,8-diol</td>
<td>2.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>5-MeC-1,2-diol</td>
<td>7.2</td>
<td>199 ± 28</td>
<td>373 ± 63</td>
</tr>
<tr>
<td>F</td>
<td>5-Hydroxymethylchrysene</td>
<td>7.3</td>
<td>136 ± 14</td>
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</tr>
<tr>
<td>G</td>
<td>9-OH-5-MeC</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>7-OH-5-MeC</td>
<td>6.1</td>
<td>148 ± 15</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1-OH-5-MeC</td>
<td>2.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*See Chart 2.*

a From 103 nmol 5-MeC, 5.4 mg protein, 20 min.

b Values observed without subtraction of negative control.

c From 3.10 µmol 5-MeC. Negative control, DMSO, 100 ±5 revertants/plate.

d From 6.20 µmol 5-MeC. Negative control, DMSO, 86 ± 15 revertants/plate.

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<td>I</td>
<td>1-OH-5-MeC</td>
<td>2.6</td>
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</tbody>
</table>

*a Tentative identifications (see text).
formation of 5-MeC-1,2-diol over 5-MeC-7,8-diol. Clearly, the degree to which potentially mutagenic or carcinogenic diols are actually formed from polynuclear aromatic hydrocarbons is critical in determining the activity of the parent compound. The structural factors influencing formation of these proximate metabolites are currently under study.

REFERENCES


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