Identification of Mutagenic Metabolites of Indeno[1,2,3-cd]pyrene Formed in Vitro with Rat Liver Enzymes


ABSTRACT

Indeno[1,2,3-cd]pyrene (IP) is a major environmental pollutant which is carcinogenic on mouse skin and in rat lung. Unlike benzo(a)pyrene, IP is a nonalternant polycyclic aromatic hydrocarbon which is devoid of a bay region. IP was mutagenic in Salmonella typhimurium TA100 in the presence of a 9000 × g supernatant from the livers of Aroclor-pretreated rats. Using a similar activation system, the major metabolites of IP were isolated and identified by comparison with synthetic reference standards. trans-1,2-Dihydro-1,2-dihydroxy-IP, 8-, 9-, and 10-hydroxy-IP, 8- and 9-hydroxy-trans-1,2-dihydro-1,2-dihydroxy-IP, and IP-1,2-quinone are among the metabolites formed in vitro. The 1,2-epoxide of indeno[1,2,3-cd]pyrene is a potent direct-acting mutagen. 8- and 9-hydroxy-IP were mutagenic with metabolic activation. 1-, 2-, and 6-hydroxy-IP and the trans-1,2-dihydrodiol had no significant mutagenic activity in S. typhimurium TA100 with metabolic activation. These data suggest that the K-region oxides of IP and of 8- and 9-hydroxy-IP are ultimately responsible for its mutagenic activity.

INTRODUCTION

IP3 (Chart 1) is a nonalternant polycyclic aromatic hydrocarbon (2) which, like other PAH, is formed primarily by the incomplete combustion of organic matter. IP, which is one of the more prevalent PAH found in the human environment, has been detected in cigarette smoke condensate, automobile and diesel engine exhaust, coal-derived oils, river sediments, charcoal-broiled foods, and ground water (3–8). Analysis for IP has been recommended by the WHO European Standards for Drinking Water and this hydrocarbon is listed as a priority pollutant by the Environmental Protection Agency.

Previous studies have shown IP to be a weak tumor initiator and a complete carcinogen on mouse skin (9, 10). IP is also active as a carcinogen in rat lung (11, 12). Despite the ubiquitous occurrence of this compound and its demonstrated biological activity, no studies have been performed to date on its metabolic activation. The objectives of this study are to determine the major metabolites of IP as formed in vitro with rat liver enzymes and determine the mutagenic potency of identified metabolites.

MATERIALS AND METHODS

Instrumentation. HPLC analyses were performed using a Waters Associates, Inc., Model ALC/GPC-204 high-speed liquid chromatograph equipped with a Model 6000A solvent delivery system, an automated gradient controller, a Model 440 UV-visible detector monitoring at 254 nm, and a Model U6K septumless injector and using a Vydac 10-μm reverse-phase C8 column, 4.6 mm x 25 cm or a Vydac 10-μm semipreparative reverse-phase C8 column, 10 mm x 25 cm (The SEP/A/RA/TIONS Group). UV spectra were recorded on a Cary Model 118 spectrophotometer and were measured in methanol or methanol:water. Mass spectra were recorded with a Hewlett-Packard Model 5982A instrument.

Chemicals. IP was purchased from the Commission of the European Communities Community Bureau of Reference, Brussels, Belgium, and from CTC Organics, Atlanta, GA. [G-3H]-Indeno[1,2,3-cd]pyrene (specific activity, 2.37 Ci/mmol) was obtained from Midwest Research Institute. cis-1,2-Dihydro-1,2-dihydroxy-IP was prepared from IP by treatment with osmium tetroxide. Oxidation with manganese dioxide afforded IP-1,2-oxide which was reduced with potassium borohydride to trans-1,2-dihydro-1,2-dihydroxy-IP (13). 1- and 2-hydroxy-IP were prepared from the cis-dihydroxy IP by treatment with tetrabutylammonium hydroxide and phosphoric acid, respectively (14). 2-Fluorene carboxaldehyde reacted with benzylmagnesium chloride to give an alcohol which was dehydrated in acid and photocyclized yielding 13H-indeno[1,2-c]-phenanthrene. 6-Hydroxy-IP was prepared from 13H-indeno[1,2-c]-phenanthrene by treatment with n-butyl lithium and carbon dioxide followed by acid-catalyzed cyclization. Diels-Alder reaction of cyclopenta[cd]pyrene (15) with 1-acetoxy-1,3-butadiene, followed by hydrolysis and oxidation with pyridinium chlorochromate, afforded a mixture of 7- and 10-hydroxy-IP. Similarly, reaction of 2-trimethylsilyloxy-1,3-butadiene with cyclopenta[cd]pyrene yielded after hydrolysis and aromatization a mixture of 8- and 9-hydroxy-IP. Unambiguous synthesis of 7- and 8-hydroxy-IP was performed by the modified Hey-Gomberg coupling (16) of either 3-methoxy-2-nitroaniline (17) or 4-methoxy-2-nitroaniline to position 1 of pyrene. Catalytic reduction of the nitro group followed by copper-catalyzed diazo coupling of the amino group to position 4 of pyrene afforded either 7- or 8-methoxy-IP. The corresponding phenols were obtained upon treatment of the methoxy compounds with boron tribromide. IP-1,2-oxide was prepared from the cis-dihydroxy by the method of Dansette and Jerina (18). The cis-dihydrodiol was converted to a 1,3-dioxolane upon treatment with trimethyl orthoacetate. Reaction with chlorotrimethylsilane in the presence of triethylamine afforded the trans-chloroacetate which was cyclized to the arene oxide with sodium methoxide. The structure of each synthetic compound was confirmed by the use of either 250- or 300-MHz nuclear magnetic resonance in extensive homonuclear spin decoupling experiments. Details of these syntheses will be published elsewhere. All of the compounds used for bioassay and metabolism studies had purities >99% as determined by HPLC analysis.

Metabolism in Vitro with Rat Liver Homogenate. In vitro metabolism studies were performed by dissolving 400 μg of IP in 200 μl of dimethyl sulfoxide and adding this solution to 4 ml of S-9 mix in a 25-ml Erlenmeyer flask. The mixture was shaken for 20 min at 37°C in a Dubnoff metabolic flask.
shaking incubator. Incubations were terminated by the addition of 2 ml of ice-cold acetone. The mixture was extracted five times with 10-ml portions of ethyl acetate which were combined, dried over sodium sulfate, and evaporated under reduced pressure below 40°C. Quantitative data were obtained by performing the incubations as above with the use of 400 μg of [3H]IP (specific activity, 1.38 mCi/mmol) followed by analysis as described below.

The S-9 mix used in these metabolism studies was prepared from the livers of male Fischer 344 rats. The rats weighed between 250 and 300 g and were given injections 5 days prior to sacrifice with Aroclor 1254 (500 mg/kg body weight). The S-9 fraction was obtained as described previously (19). Each ml of S-9 mix contained 50 μmol of potassium phosphate buffer (pH 7.4), 8.0 μmol of MgCl₂, 33 μmol of KCl, 5.0 μmol of glucose 6-phosphate, 4.0 μmol NADP+, and 0.5 ml of the S-9 fraction of the rat liver homogenate. The protein content of each milliliter of S-9 mix was 30 mg.

Analysis of Metabolites. Metabolites were separated by HPLC using a solvent program of 50% aqueous methanol for 10 min followed by a linear gradient of 50 to 65% methanol over 15 min, 65 to 85% methanol in 10 min, and 85 to 100% methanol in 20 min. For metabolism studies using [3H]IP, fractions were collected at 10-s intervals, mixed with 17 ml of Monofluor (National Diagnostics, Somerville, NJ), and analyzed for tritium content by liquid scintillation counting. Metabolites were collected from HPLC and analyzed directly by UV spectroscopy or concentrated for mass spectral analysis.

Mutagenicity Assays. The mutagenic activity of IP and IP metabolites was evaluated in Salmonella typhimurium TA100 using the procedure of Ames (19). Briefly, the compound to be tested was dissolved in 50 μl of dimethyl sulfoxide and added to 0.1 ml of an overnight culture broth of S. typhimurium TA100. After the addition of 2 ml of molten top agar at 45°C, the contents were mixed and poured onto minimal glucose agar plates. Those assays performed with metabolic activation contained 200 μl of the identical S-9 mixture used in the metabolism studies.

RESULTS

A HPLC profile of the ethyl acetate extract from the in vitro metabolism of IP is presented in Chart 2. Peak C, which was the major metabolite of IP from rat liver homogenate, was shown by its mass spectrum to be a dihydrodiol. The UV spectrum of this metabolite resembled the spectrum of benzo(b)fluoranthene suggesting this to be the 1,2-dihydrodiol of IP. This tentative assignment was confirmed by comparison of the UV and mass spectra of Metabolite C (Chart 3) and HPLC retention time with those of synthetic trans-1,2-dihydro-1,2-dihydroxy-IP. Peaks A and B had mass spectra consistent with hydroxylated dihydrodiols of IP. The UV spectrum of Peak A resembled 6-hydroxybenzo(b)fluoranthene while that of Peak B resembled 5-hydroxybenzo(b)fluoranthene (Chart 4). Incubation of 8- and 9-hydroxy-IP with the identical rat liver homogenate used above for metabolism studies of IP resulted in the appearance of Peaks A and B, respectively. Both A and B were also formed upon incubation of
MUTAGENIC METABOLITES OF IP

Table 1

<table>
<thead>
<tr>
<th>Peak</th>
<th>HPLC retention time (min)</th>
<th>UV (nm)</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>9.0</td>
<td>370, 351, 337, 308, 296, 267, 257</td>
</tr>
<tr>
<td>B</td>
<td>15.0</td>
<td>374, 355, 310, 299, 285, 266</td>
</tr>
<tr>
<td>C</td>
<td>34.5</td>
<td>373, 354, 344, 302, 292, 283, 257, 244</td>
</tr>
<tr>
<td></td>
<td>(34.5)</td>
<td>373, 354, 344, 303, 292, 282, 257, 242a</td>
</tr>
<tr>
<td>D</td>
<td>42.0</td>
<td>345, 326, 248</td>
</tr>
<tr>
<td></td>
<td>(42.0)</td>
<td>345, 330, 248</td>
</tr>
<tr>
<td>E</td>
<td>46.0</td>
<td>376, 356, 306, 278, 251, 218</td>
</tr>
<tr>
<td>F</td>
<td>47.2</td>
<td>373, 354, 321, 309, 298, 280, 272, 250</td>
</tr>
<tr>
<td></td>
<td>(47.2)</td>
<td>374, 355, 322, 310, 298, 281, 272, 251</td>
</tr>
<tr>
<td>G</td>
<td>48.3</td>
<td>338, 321, 308, 296, 274, 250, 240</td>
</tr>
<tr>
<td>H</td>
<td>50.0</td>
<td>390, 370, 354, 318, 305, 280, 252, 217</td>
</tr>
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<td></td>
<td>(50.0)</td>
<td>390, 370, 353, 319, 306, 281, 253, 217</td>
</tr>
<tr>
<td>I</td>
<td>52.0</td>
<td>394, 385, 350, 314, 299, 250, 234, 224</td>
</tr>
<tr>
<td></td>
<td>(52.0)</td>
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</tr>
<tr>
<td>J</td>
<td>56.0</td>
<td>320, 296, 283, 248, 240</td>
</tr>
</tbody>
</table>

a The numbers in parentheses are the values obtained from synthetic reference samples, where available.

Chart 4. UV spectra of Metabolites A and B compared with spectra of 5- and 6-hydroxybenzo[a]fluoranthene.

IP-1,2-dihydridiol under identical conditions. On the basis of these data, Metabolites A and B were identified as 8-hydroxy-IP, 1,2-dihydridiol and 9-hydroxy-IP, 1,2-dihydridiol, respectively. Peak D was identified as IP-1,2-quinone by comparison of its UV spectrum and HPLC retention time with those of a synthetic sample. This quinone was also shown to be formed by autoxidation of the trans-1,2-dihydridiol and 1- and 2-hydroxy-IP. The presence of this quinone among the metabolites of IP is most probably due to decomposition of the trans-1,2-dihydridiol. Metabolites F and H, which are the major phenolic metabolites of IP, were shown to be 8- and 9-hydroxy-IP, respectively, by comparison of their UV and mass spectra and HPLC retention time with synthetic samples. Similarly, Metabolite I was identified as 10-hydroxy-IP by comparison of its HPLC retention time and spectral data with a synthetic sample. Neither 1-, nor 2-, nor 6-hydroxy-IP were detected among the metabolites of IP formed in vitro in rat liver homogenate. No oxygenated metabolites of IP were detected when IP was incubated with heat-deactivated S-9 mix. HPLC retention times and UV spectral data for the metabolites of IP are summarized in Table 1.

Quantitation of metabolites was performed using [3H]IP in rat liver homogenate. [3H]IP was metabolized to the extent of 81% in rat liver homogenate. The trans-1,2-dihydridiol of IP comprised 30% of the ethyl acetate-extractable metabolites. IP-1,2-quinone was formed to the extent of only 3% of the ethyl acetate-extractable metabolites of IP. 8-Hydroxy-IP, 1,2-dihydridiol and 9-hydroxy-IP, 1,2-dihydridiol accounted for 21 and 10%, respectively, of the ethyl acetate-extractable metabolites. Among the phenolic metabolites, 8-hydroxy-IP, 9-hydroxy-IP, and 10-hydroxy-IP comprised 7, 8, and 5%, respectively, of the ethyl acetate-extractable metabolites. No attempt was made to quantitate any water-soluble metabolites of IP formed using rat liver homogenate.

IP was found to be mutagenic in S. typhimurium TA100 with metabolic activation using the identical S-9 mix used in the in vitro metabolism studies. In the absence of S-9 mix IP is inactive as a mutagen. The trans-1,2-dihydridiol was not a major mutagenic metabolite of IP as is shown in Chart 5. 8- and 9-hydroxy-IP were both strongly mutagenic when assayed in the presence of S-9 mix. Neither 1-, nor 2-, nor 6-hydroxy-IP were found to be strongly mutagenic when assayed under identical conditions. IP-1,2-oxide was shown to exhibit potent mutagenic activity when assayed in the absence of metabolic activation (Chart 6).
Among the metabolites of IP which have been tested for mutagenic activity, only 8- and 9-hydroxy-IP were active when assayed with metabolic activation. 9-Hydroxy-IP displayed a sharp linear increase in mutagenic activity with respect to dose at very low doses, after which the mutagenic activity remained relatively constant. One explanation for this effect might be that 9-hydroxy-IP is metabolized to an electrophile more efficiently at high S-9 mix:substrate ratios. Neither 8- nor 9-hydroxy-IP displayed any direct-acting mutagenic activity in the tester strain used. In view of the abundance of the \textit{trans}-1,2-dihydrodiols of these phenols among the metabolites of IP, it is likely that the epoxide precursors to these phenolic dihydrodiols contribute to the overall mutagenic activity of IP. The formation of phenolic epoxides as electrophilic metabolites of a PAH is not without precedent. 9-Hydroxybenzo(a)pyrene-4,5-oxide has been shown to bind to DNA both \textit{in vitro} and \textit{in vivo} (29–34). Although 9-hydroxybenzo(a)pyrene was found to be more mutagenic in \textit{S. typhimurium} than benzo(a)pyrene when assayed in the presence of rat liver microsomes (35), it is inactive as a complete carcinogen on mouse skin (36) and only weakly active as a tumor initiator in the same target tissue (37). A phenolic diol-epoxide of chrysene has recently been reported to form an adduct with DNA \textit{in vivo} in mouse skin (38, 39). This adduct comprises approximately 50% of the chrysenol:DNA adducts formed in that tissue. Although no tumor data have been reported, the phenol from which this triol-epoxide is derived, 3-hydroxychrysene, is mutagenic in \textit{S. typhimurium} when assayed with metabolic activation (38, 39). The contribution of 8-hydroxy-IP to the overall tumorigenicity of IP is currently being evaluated on mouse skin.

REFERENCES

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