Transformation of pyrene in aqueous chlorination in the presence and absence of bromide ion: Kinetics, Products and Their Aryl Hydrocarbon Receptor-Mediated Activities

Jianying Hu¹*, Xiaohui Jin¹, Shoichi Kunikane², Yoshiyasu Terao³, Takako Aizawa⁴

¹College of Environmental Science, Peking University, Beijing, 100871, China

²Department of Water Supply Engineering, National Institute of Public Health, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108, Japan

³Institute for Environmental Sciences, University of Shizuoka, 52-1, Yada, Shizuoka 422-8526, Japan

⁴Yokohama Waterworks Bureau, 522 Kawashima-cho, Hodogaya-ku, 240-0045, Japan
**Kinetics Experiments.** Tested aqueous solutions (200mL) were buffered using phosphate salts for 5.5-9.0 pH range. For pH < 5.5 and pH > 9, pH values were preadjusted with H$_2$SO$_4$ and NaOH, respectively. The initial pyrene concentration was 0.5 µM, at least 35 µM of total chlorine was added. Chlorine variation was less than 5% under these conditions. The chlorine concentration was thus assumed to be constant during the kinetics experiments.

Kinetic runs were initiated by injecting, under rapid mixing, an aliquot of sodium hypochlorite solution. At different reaction times, 3 ml of solution was rapidly transferred into a vial containing 100 µL of sodium thiosulfate solution (1 M) to quench the residual chlorine and stop the reaction. Samples were then analyzed using HPLC to determine the remaining pyrene concentration. When the pyrene disappeared, the kinetic experiments were pursued until at least 50% pyrene consumption was achieved.

Each sample was analyzed by HPLC with a reversed-phase C18 column (Zorbax-ODS, 4.6 mm I.D. × 250 mm in length, 5 µm in particle diameter, Alliance and Agilent, USA) at 25°C. The initial mobile phase composition was acetonitrile /water (70/30 v/v), which was increased linearly to 100% acetonitrile in 10 min., and then held for 15 min. The flow rate, UV-detection wavelength, and sample injection volume were 1.5 mL/min, 333 nm, and 50 µL, respectively.

**Identification and analysis of by-products.** The experiments were carried out in a glass reactor which was placed in a water bath to maintain the reaction temperature at 25°C. Synthetic raw water was prepared by dissolving 0.25 mg of a standard pyrene into 2.5 L Milli-Q pure water of which the pH was adjusted to 7.22 by adding phosphate buffer. A 500-mL sample was removed for determination of the aryl hydrocarbon receptor mediated activity before NaOCl was added to the remaining solution. Samples (500 mL) were taken out at different chlorination time after NaOCl was added to the remaining solution ([HOCl]$_T$ = 35 µM). After decomposition of the residual HOCl by the addition of Na$_2$S$_2$O$_3$ (1 M) and after the pH was adjusted to 2-3, the samples were concentrated by solid phase extraction (SPE, Waters Sep-Pak C18, Waters, USA). The cartridge was conditioned with 5ml dichloromethane, 5ml methanol and 5ml water. Samples
were passed through the cartridge at a flow rate of 10-15 mL/min. Additional water (10mL) was applied to wash the wall of the cartridge. The residual water was removed by passing a gentle nitrogen stream through the cartridge for about 30 min. 10mL dichloromethane was percolated at 4-5mL/min through the sorbent bed to mobilize pyrene and its chlorinated products. Of the 10mL eluant, 8mL was dried under a gentle nitrogen stream, and redissolved in 0.2mL hexane for GC-MS analysis. The rest of the eluant (2mL) was dried and redissolved in 0.02mL DMSO to assess the AhR-mediated activity using the yeast two-hybrid assay. The products in an aqueous chlorinated solution of pyrene were analyzed by GC-MS (Hewlett-Packard (5890-5971)), and identified by NMR.

The column was a HP-5MS (60m × 0.32mm × 0.25µm, J & W Scientific, USA). Splitless injections were employed with 5-psi head pressure and 2 mL/min He carrier flow rate. The temperature was increased from 70°C to 210°C at the rate of 15°C/min, then raised to 300°C at the rate of 8°C /min (and held for 10 min) for analysis of the aqueous chlorinated samples without the addition of bromide ion. For the analysis of samples with the addition of bromide ion, the temperature program was 70°C to 300°C at the rate of 8°C /min (held for 10 min). The mass range scanned was 50 to 550 amu with a scan time of 1.6 sec.

The $^1$H NMR and H-H COSY spectra were measured on a Bruker ARX400 ($^1$H, 400 MHz, Switzerland) instrument. Deuterochloroform was used as the solvent. The chemical shifts (δ values) are given in ppm downfield from tetramethylsilane.

**Synthesis of 1,8-dibromopyrene and 1,6-dibromopyrene.** Di-Br-pyrene standards were obtained by the reaction of 1-bromopyrene with sodium hypochlorite in the presence of potassium bromide in acidic 50% methanol solution. This product was suggested to be a mixture of two isomers by GC/MS analysis. Separation of two isomers was successful by following fractional recrystallization. The mixture was dissolved in hot hexane and allowed to stand at room temperature to deposit colorless crystals, which were collected on a suction filter. These crystals were 1,8-dibromopyrene, mp 206-208 °C. The residual solid obtained by concentration of the
filtrate was recrystallized twice from hexane to give pure crystals of 1,6-dibromopyrene, mp 224-227 °C.

**Yeast Assay for AhR-mediated Activity of Products.** The yeast strain YCM3 with human AhR, Arnt and the LacZ reporter plasmid, pTXRE5-Z, was used to test the AhR-mediated activity. The human AhR and Arnt genes are integrated into chromosome III. AhR and Arnt are expressed from the galactose-regulated GAL 1,10 promoter. Transcriptional activation mediated by the AhR/Arnt heterodimer is assessed by β-galactosidase activity. Expression of the LacZ reporter plasmid, TXRE5-Z, is directed by the AhR-Arnt complex binding to five response elements in the promoter region. The yeast cells were preincubated at 30°C for 22 hours in 5 mL medium (6.7 g/L Difco yeast nitrogen base without amino acids, 0.2% glucose, 117.6 mg/L L-Leucine, 300 mg/L L-isoleucine, 1500 mg/L L-valine, 200 mg/L L-adenine hemisulfate salt, 200 mg/L L-arginine HCl, 200 mg/L L-histidine HCl monohydrate, 300 mg/L L-lysine HCl, 200mg/L L-methionine, 500mg/L L-phenylalanine, 200 mg/L L-threonine, 300 mg/L L-tyrosine, 200mg/L L-uracil (Sigma, USA)). 50 µL of overnight culture and 2.5 µL of DMSO solution diluted to the desired concentrations were then added to 200 µL of fresh medium (2% galactose) in a glass tube (10 mm × 50 mm), respectively. After yeasts were cultured for 8 h at 30°C, 150 µL of the above culture was fractionated, and its absorbance at 595 nm was detected. The residual culture (100 µL) was centrifuged at 4 °C (15000 rpm) for 5 min, and the collected cells were resuspended in 200 µL of Z buffer (0.1 M sodium phosphate (pH = 7.0), 10 mM KCl, 1 mM MgSO₄) containing 1mg/mL Zymolyase 20T (Seikagaku, Tokyo), and incubated for 20 min at 30°C. The enzymatic reaction was started by the addition of 40 µL of 4 mg/mL 2-nitrophenyl-β-D-galactopyranoside (ONPG, Tokyo Kasei, Tokyo, Japan), and incubated for 20 min at 30°C. Then the enzymatic reaction was stopped by adding 1 M Na₂CO₃ (100 µL). After the above solution was centrifuged, 150-µL aliquots were placed into 96 wells of a microplate. Absorbances at 415 and 570nm were read on a microplate reader (Bio RAD 550, USA) to estimate the AhR-mediated activity, and the β-galactosidase activity (U) was calculated according to Equation (1):
where \( t \) represents the reaction time (min), \( v \) is the volume of the culture used in the assay (mL), \( \text{OD}_{595} \) is the cell density at the start of the assay, \( \text{OD}_{415} \) is the absorbance by o-nitrophenol at the end of the reaction, and \( \text{OD}_{570} \) is the light scattering at the end of the reaction. In this assay, \( \beta \)-naphthoflavone (Chemservice, Chester, England) was used as positive control.

**Kinetics of chlorination of pyrene.** The kinetics of pyrene chlorination were investigated under conditions for pseudo-first-order kinetics \( ([\text{HOCl}]_0 > 20 \times [\text{pyrene}]_0) \). The initial concentration of pyrene was 0.5 \( \mu \text{M} \) and at least 35 \( \mu \text{M} \) of chlorine was added. Chlorine variation was less than 5% under these conditions. The concentration of chlorine was assumed to be constant during the reaction time. Figure 1 shows the plot of Ln([pyrene]_t/[pyrene]_0) as a function of reaction time at pH = 5.01, 6.57 and 7.22. The results show that the kinetic experiments can be well described by a pseudo-first-order kinetics as demonstrated by the linear plots \( (r^2 > 0.97) \). The reaction order with respect to HOCl was examined at pH 7.22 by varying its concentration. The insert in Figure 1 shows that the pseudo-first-order rate constant, \( k_{\text{obs}} \), is linearly correlated to the chlorine dose. Therefore, the rate of pyrene disappearance is first order to total concentration of chlorine \( ([\text{HOCl}]_T = [\text{HOCl}] + [\text{OCl}^-]) \) and to the pyrene concentration \( ([\text{pyrene}]) \):

\[
\frac{d[\text{pyrene}]}{dt} = k_{\text{app}}^{\text{Cl}}[\text{pyrene}][\text{HOCl}]_T
\]

with \( k_{\text{app}}^{\text{Cl}} = k_{\text{obs}} /[\text{HOCl}]_T \) is a second-order kinetic constant.

The pyrene chlorination kinetics can be expressed as follows:

\[
\text{HOCl} + \text{H}^+ + \text{OCl}^- \rightarrow \text{products} \quad k_1
\]

\[
\text{pyrene} + \text{HOCl} \rightarrow \text{products} \quad k_2
\]

and the rate expression for the above reaction is
\[
v = -\frac{d[\text{pyrene}]}{dt} = k_1[\text{HOCl}][\text{pyrene}] + k_2[\text{OCl}^-][\text{pyrene}]
\]

By replacing \([\text{HOCl}]\) and \([\text{OCl}^-]\) as ratios of \([\text{HOCl}]_T\), the rate of pyrene disappearance is

\[
v = -\frac{d[\text{pyrene}]}{dt} = k_1\alpha_1^{\text{Cl}}[\text{HOCl}]_T[\text{pyrene}] + k_2\alpha_2^{\text{Cl}}[\text{HOCl}]_T[\text{pyrene}]
\]

where \(\alpha_i^{\text{Cl}}\) is the ionization fraction of hypochlorous acid species, with \(i = 1\) or \(2\), for \(\text{HOCl}\) and \(\text{OCl}^-\), respectively.

Therefore, by combining Eqs (2) and (7), \(k_{\text{app}}^{\text{Cl}}\) is a function of pH

\[
k_{\text{app}}^{\text{Cl}} = k_1\alpha_1^{\text{Cl}} + k_2\alpha_2^{\text{Cl}} = k_1\frac{[\text{H}^+]}{K_{a_{\text{Cl}}} + [\text{H}^+]} + k_2\frac{K_{a_{\text{Cl}}}}{K_{a_{\text{Cl}}} + [\text{H}^+]}
\]

In order to investigate the effect of bromide ion on the kinetics of reaction, 5 \(\mu\text{M}\) bromide ions were added in water before chlorination reaction. Hypobromous acid (\(\text{HOBr}\)) is formed because of reaction with chlorine (\(\text{HOCl} + \text{Br}^- \rightarrow \text{HOBr} + \text{Cl}^-\)). This reaction is fast. So equal molar of hypobromous acid (equal to the concentration of bromide ions added) was formed. The experimental procedure was as the same as described above. Figure 2 shows the plot of \(\ln([\text{pyrene}]_t/[\text{pyrene}]_0)\) as a function of reaction time at pH = 6.80, 7.82 and 8.27. The results show that the kinetic experiments can be well described by a pseudo-first-order kinetics as demonstrated by the linear plots \(r^2 > 0.96\). The reaction order with respect to bromide ions was examined at pH 8.27 by varying its concentration. The insert in Figure 2 shows that the pseudo-first-order rate constant, \(k_{\text{obs}}\), is linearly correlated to the concentration of total concentration of hypobromous acid and the existence of \(\text{HOCl}\) do not significantly contribute to the overall reaction rate. Therefore, the rate of pyrene disappearance is first order to concentration of hypobromous acid (\([\text{HOBr}]_T = [\text{HOBr}] + [\text{OBr}^-]\)) and to the pyrene concentration (\([\text{pyrene}]\)):

\[
v = -\frac{d[\text{pyrene}]}{dt} = k_{\text{obs}}[\text{pyrene}] = k_{\text{app}}[\text{HOBr}]_T[\text{pyrene}]
\]

The reactions in the presence of bromide ions can be expressed as follows:
\[ \begin{align*}
1 & \quad \text{HOCl} + \text{Br}^- \rightarrow \text{HOBr} + \text{Cl}^- \quad (10) \\
2 & \quad \text{HOBr} \rightarrow \text{H}^+ + \text{OBr}^- \quad \text{Ka}_{\text{br}} \quad (11) \\
3 & \quad \text{pyrene} + \text{HOBr} \rightarrow \text{products} \quad k_3 \quad (12) \\
4 & \quad \text{pyrene} + \text{OBr}^- \rightarrow \text{products} \quad k_4 \quad (13) \\
5 & \quad \text{The rate expression can be written as follows:} \\
6 & \quad v = k_3[\text{pyrene}][\text{HOBr}] + k_4[\text{pyrene}][\text{OBr}^-] \quad (14) \\
7 & \quad \text{By replacing [HOBr] and [OBr] as ratios of [HOBr]_T, the rate of pyrene disappearance is} \\
8 & \quad v = -\frac{d[\text{pyrene}]}{dt} = k_3\alpha_{i}^{\text{br}}[\text{HOBr}]_T[\text{pyrene}] + k_4\alpha_{2}^{\text{br}}[\text{HOBr}]_T[\text{pyrene}] \quad (15) \\
9 & \quad \text{where } \alpha_{i}^{\text{br}} \text{ is the ionization fraction of hypochlorous acid species, with } i = 1 \text{ or } 2, \text{ for HOBr and OBr}^{-}, \text{ respectively.} \\
10 & \quad \text{Therefore, by combining Eqs (9) and (15), } k_{\text{app}}^{\text{br}} \text{ is a function of pH} \\
11 & \quad k_{\text{app}}^{\text{br}} = k_3\alpha_{1}^{\text{br}} + k_4\alpha_{2}^{\text{br}} = k_3 \frac{[\text{H}^+]}{\text{Ka}_{\text{br}} + [\text{H}^+]} + k_4 \frac{\text{Ka}_{\text{br}}}{\text{Ka}_{\text{br}} + [\text{H}^+]} \quad (16) \\
12 & \quad \text{Calculation of Model Selection Criterion (MSC). We employed MSC as an index to estimate} \\
13 & \quad \text{the goodness of fit for the regressions in Figure 1. The MSC is an extension of Akaike} \\
14 & \quad \text{information criterion (AIC), which is derived from the maximum likelihood function (1). The} \\
15 & \quad \text{MSC is estimated as following equation:} \\
16 & \quad \ln \left[ \frac{\sum_{i=1}^{n} W_i (x_i - \bar{x}_i)^2}{\sum_{i=1}^{n} W_i (x_i - \bar{x}_i)^2} \right] - \frac{2p}{n} \\
17 & \quad \text{where } x_i \text{ represents the i-th observed data, } \bar{x}_i \text{ is the i-th predicted value, } \bar{x}_i \text{ is the mean} \\
18 & \quad \text{observed value, } n \text{ is the number of data, } p \text{ is the number of parameters, and } W_i \text{ is the weighting} \\
19 & \quad \text{of data. The value of } W_i \text{ depends on the uncertainty of data. In general, the } W_i \text{ should be 1. The} \\
20 & \quad \text{higher the MSC, the closer the model explains the observed values. The theoretical models that} \\
21 & \quad \text{produce MSC values larger than 3 are regarded to exhibit an acceptable fit to data, whereas} \\
\end{align*} \]
exceptionally good fit (MSC>6) should be taken as suspects, and the value less than 2 was regarded unacceptable. As a result, the MSCs for the regressions (Figure 1) in the absence and presence of bromide ions were 3.10 and 3.85, respectively.

Literature Cited

**SUPPORTING INFORMATION Table 1.** GC/MS mass spectra of products in aqueous chlorinated solution of pyrene.

<table>
<thead>
<tr>
<th></th>
<th>Retention time</th>
<th>Molecular ion</th>
<th>m/z (relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pyrene</td>
<td>17.287</td>
<td>202</td>
<td>202(100), 203(17), 200(15), 201(14), 101(99)</td>
</tr>
<tr>
<td>1-Cl-pyrene</td>
<td>19.530</td>
<td>236</td>
<td>236(100), 238(32), 200(30), 100(21), 235(18)</td>
</tr>
<tr>
<td>1-Br-pyrene</td>
<td>20.714</td>
<td>280</td>
<td>280(100), 282(98), 201(68), 200(65), 100(51)</td>
</tr>
<tr>
<td>di-Cl-pyrene</td>
<td>21.408</td>
<td>270</td>
<td>270(100), 272(66), 200(45), 100(20), 274(10)</td>
</tr>
<tr>
<td></td>
<td>21.728</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td>Br-Cl-pyrene</td>
<td>22.809</td>
<td>316</td>
<td>314(100), 316(75), 200(61), 100(35), 101(20)</td>
</tr>
<tr>
<td></td>
<td>23.169</td>
<td>316</td>
<td></td>
</tr>
<tr>
<td>di-Br-pyrene</td>
<td>24.369*</td>
<td>360</td>
<td>360(100), 362(52), 358(49), 281(25), 200(69)</td>
</tr>
<tr>
<td></td>
<td>24.769*</td>
<td>360</td>
<td></td>
</tr>
</tbody>
</table>

* Retention time in Figure 3.
SUPPORTING INFORMATION Figure 1. Pseudo-first-order kinetic plot of pyrene chlorination at 20 ± 2 °C, [HOCl]_T = 35 μM and three pH levels. Symbols represent measured data, and the straight line is the linear regression. (Insert: pH = 7.22, 20 ± 2 °C, and various [HOCl]_T)
SUPPORTING INFORMATION Figure 2. Pseudo-first-order kinetic plot of pyrene chlorination at 20 ± 2 °C, [HOCl]_T = 35 µM and three pH levels with addition of bromide ions (5 µM). Symbols represent measured data, and the straight line is the linear regression. (Insert: pH = 9.20, 20 ± 2 °C, [HOCl]_T = 35 µM and various [Br^-]).
SUPPORTING INFORMATION Figure 3. Atom HOMO (highest occupied molecular orbit) density of pyrene and 1-Br-pyrene. (a) pyrene; (b) 1-Br-pyrene. Atom HOMO density were calculated by MOPAC Ver.6 (CAChe Scientific Inc., Oxford)