Nitroarene Concentrations and Direct-Acting Mutagenicity of Diesel Exhaust Particulates Fractionated by Silica-Gel Column Chromatography

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Diesel exhaust particulates were extracted with benzene–ethanol (3:1, v/v) and separated into five fractions by silica-gel column chromatography. Direct-acting mutagenic activity was assayed by the Ames test using the Salmonella typhimurium YG1024 strain. The total activity of five fractions was about four times greater than that of the crude extract, suggesting that the activities in the fractions were suppressed in the crude extract. Strong activity was observed in fraction 4 which was eluted with dichloromethane (61.5% of the total activity) and fraction 5 which was eluted with ethanol (35.3%). Nitropoly cyclic aromatic hydrocarbons (NPAs) were determined by high-performance liquid chromatography with chemiluminescence detection. They were found mainly in fraction 4, although one NPA was in fraction 3 which was eluted with n-hexane–dichloromethane (3:1, v/v). Based on these results, 53.1% of the activity in fraction 4 was attributed to NPAs. The contribution of 1-nitropyrene and 1,3-, 1,6- and 1,8-dinitropyrenes was great and that of the other NPAs was small. The mutagenic compound in fraction 5 was not identified. Fractions 1 and 2, which were eluted with n-hexane, and fraction 3 suppressed the activity of fraction 4. Polycyclic aromatic hydrocarbons in fractions 2 and 3 were considered as possible suppressors of NPAs.

Key words nitroarene; diesel exhaust particulate; direct-acting mutagenicity; silica-gel column chromatography

Diesel exhaust particulates (DEP) have been considered to be a cause of lung cancer. Previous studies have shown that extracts from DEP obtained with organic solvents contained carcinogens and mutagens such as polycyclic aromatic hydrocarbons (PAHs) and nitropoly cyclic aromatic hydrocarbons (NPAs). Many studies have been done on PAHs in DEP as well as in airborne particulates. However, studies on the behavior and mutagenic contribution of NPAs have been limited because of their low concentrations. However, the mutagenicity of DEP and airborne particulates has been assayed by the Ames test using strains of Salmonella typhimurium, and the mutagenic contribution of several NPAs has been reported.1–5) Of the NPAs, 1,8-dinitropyrene (1,8-DNP) showed the strongest direct-acting mutagenicity.6)

We have developed a high-performance liquid chromatographic (HPLC) method for 1,3-, 1,6- and 1,8-DNPs and 1-nitropyrene (1-NP) using chemiluminescence detection7,8) and recently determined their concentrations in DEP and airborne particulates.9–11) When airborne particulates were separated with an Andersen high-volume air sampler, the concentration of these NPAs was highest in the finest particulate fraction (<1.1 μm) in which DEP mainly collected. If the effect of coexisting compounds is assumed to be negligible, 68.6% of the mutagenicity of the benzene–ethanol extract from airborne particulates could be attributed to this fraction. In this fraction, the calculated mutagenic contribution of 1,3-, 1,6- and 1,8-DNPs and 1-NP was respectively 2.5, 5, 9 and 2.1% in the S. typhimurium YG 1024 strain.12) However, the effects of coexisting compounds on the mutagenicity have not been considered.

In this report, DEP was extracted with benzene–ethanol and thoroughly separated into five fractions by silica-gel column chromatography. We found that the direct-acting mutagenicity was suppressed in the crude extract and became stronger after fractionation. In each fraction, ten NPAs were identified and their mutagenic contribution was calculated. Also, the effects of coexisting compounds on the direct-acting mutagenicity of NPAs are discussed.

Experimental

Chemicals 1,3-, 1,6- and 1,8-DNPs and 2-fluoro-7-nitrofluorene (internal standard) were obtained from Aldrich (Milwaukee, WI, U.S.A.). 1-NP and 2-nitrofluorene were purchased from Tokyo Kasei (Tokyo, Japan). 6-Nitrobenz[a]pyrene (6-NBaP) and 3-nitropyrene (3-NP) were purchased from Chensyn Science Laboratories (Lexena, KS, U.S.A.). 2- and 4-NPs were kindly provided by Dr. Y. Hisamatsu of the National Institute of Public Health (Tokyo, Japan). 2-Nitrofluoranthene (2-NF) and 6-nitropyrene (6-NC) were kindly provided by Dr. N. Sera of the Fukushima Institute of Health and Environmental Science (Fukuo ka, Japan). Fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[ghi]perylene were purchased from Kanto Chemical (Tokyo, Japan). Nutrient broth (No. 2), glucose and agarose were purchased from Oxoid (Hamphshire, UK). Wako Pure Chemical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Dimethyl sulfoxide (DMSO) was obtained from DOJINDO Laboratory (Kumamoto, Japan). All other chemicals were commercially available, special grade.

Sampling and Extraction of DEP A diesel-engine vehicle (1995 model, 3.5 L) was used for this study. A Sibata (Tokyo, Japan) L-30 low-volume air sampler with a Millipore AP 20 glass-fiber filter was positioned 30 cm from the end of the exhaust pipe of the idling engine. Exhaust particulates were collected on the filter at a flow rate of 30 m/min and the filter was changed every 15 min. After the filters were dried in a desiccator for 2 days and weighed, they were stored in a freezer (−20 °C) until use. The collected particulates (0.38 g) were extracted twice with 50 ml of benzene–ethanol (3:1, v/v), homogenized ultrasonically and then filtered through a No. 6 filter paper (Advantec, Tokyo, Japan) (crude DEP extract solution).

Silica-Gel Column Chromatography After the crude DEP extract solution was evaporated to dryness, the residue (0.26 g) was redisolved in 0.5 ml n-hexane and applied to a silica-gel column (Wakogel Q200, 200 mesh, 20 × 120 mm bed). The column was eluted successively with n-hexane (40 + 200 ml), n-hexane–dichloromethane (3:1, v/v) (200 ml), dichloromethane (200 ml) and methanol (450 ml), and the corresponding fractions were obtained (fractions 1–5). Each fraction was divided into three equal volumes and all solutions were evaporated to dryness. Two were redisolved in 4 ml acetonitrile for the determination of NPAs and PAHs, respectively, and one in 2 ml of DMSO for the mutagenicity assay.

Determination of NPAs Ten NPAs (2-NF, 1-, 2-, 4-NPs, 6-NC, 6-NBaP, 3-NP, 1,3-, 1,6-, 1,8-DNPs) were determined as described

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previously\(^{10}\) with some modifications.\(^{13}\) The HPLC system consisted of two 880-PU pumps (Jasco, Tokyo, Japan), a Rheodyne 7125 sample injector (Cotati, CA, U.S.A.) with a 20-μl loop and an S-3400 chemiluminescence detector (Soma, Tokyo, Japan). The analytical column consisted of two Nacalai Tesque Cosmosil SC18MS (4.6 i.d. × 250 mm) columns connected in series.

The mobile phase was prepared by mixing acetonitrile and imidazole-perchloric acid (pH 7.6) (1:1, v/v). The flow rate was 1 ml min\(^{-1}\). The post-column chemiluminescence reagent solution was an acetonitrile solution containing 20 μM bis(2,4,6-trichlorophenyl)oxalate and 15 mM hydrogen peroxide. The flow rate was 1 ml min\(^{-1}\).

NPAHs in each sample solution were reduced to the corresponding amino-derivatives by refluxing with sodium hydrosulfide for 1.5 h before injection into the HPLC system.

**Determination of PAHs** The HPLC system consisted of a Jasco 880-PU pump, a Rheodyne 7125 sample injector with a 20-μl loop, a Vydac 210TP 54 analytical column (4.6 i.d. × 250 mm, The Separations Group, Hesperia, CA, U.S.A.) and a Jasco FP-920S fluorescence detector. Excitation and emission wavelengths were changed respectively from 286 and 433 nm to 266 and 402 nm at 7 min and then to 294 and 430 nm at 14 min. The mobile phase was acetonitrile–water (4:1, v/v). The flow rate was 1 ml min\(^{-1}\).

**Mutagenicity Assay** Direct-acting mutagenicity was assayed by the Ames test according to the micro-suspension technique\(^{14}\) using the *Salmonella typhimurium* YG1024 strain\(^{15}\) without S9 mix. At least two plates were used for each dose, and the mean values of revertants pmol\(^{-1}\) plate\(^{-1}\) were calculated from linear regression lines fitted to the increasing portion of the dose–response curve. DMSO was used as a solvent control, and 2-nitrofluoranthene was used as a positive control.

**Results and Discussion**

**Direct-Acting Mutagenicity** Figure 1 shows the mutagenicity of the crude extract and the five fractions obtained by silica-gel column chromatography in the YG1024 strain without S9 mix. The activities are expressed as revertants per mg of DEP. The total activity of the five fractions (10400 rev mg\(^{-1}\)) was about four times greater than that of the crude extract (2560 rev mg\(^{-1}\)). The activity of fraction 4 was 61.5% of the total of the five fractions. Fraction 5 had the next highest activity (36.5% of the total activity). The sum of these two fractions was 97.8% of the total and the activity of the other fractions was very weak. This result was reproducible in three experiments. The discrepancy between the crude extract and the chromatographed fractions suggested that the activity of some mutagens in the fractions was suppressed in the crude extract, and that silica-gel column chromatography separated the mutagens from the suppressors. Removal of the suppressors was necessary to obtain accurate results in the mutagenicity assay of environmental samples such as DEP extracts.

**NPAHs and Their Mutagenic Contribution** In our previous report, 1-NP and 1,3-, 1,6- and 1,8-DNPs in DEP were simultaneously determined.\(^{10}\) Recently, 2-NF, 2-, 4-NPs, 6-NC, 6-NBaP and 3-NPPer were also determined.\(^{15}\) All these NPAHs showed direct-acting mutagenicity. Therefore, these ten NPAHs were determined in this report.

As shown in Table 1, NPAHs were mainly eluted in fraction 4 which exhibited the strongest activity. An exception was 6-NPPer, which was eluted in fraction 3. The concentration of 1-NP was about one order of magnitude higher than that of 6-NPPer and more than one order of magnitude higher than those of the other NPAHs. The fact that neither 2-NF nor 2-NP was detected in any of the fractions suggested that these compounds were not produced in the diesel engine but formed secondarily in the atmosphere after emission.\(^{16}\)

In this study, the direct-acting mutagenicity of 1-, 4-NPs, 6-NC, 6-NBaP, 3-NPPer, 1,3-, 1,6- and 1,8-DNPs was 240, 890, 46, 23, 20, 4650, 5810 and 11570 rev pmol\(^{-1}\), respectively, in the YG1024 strain. By substituting these values into Table 1, the mutagenic contributions of these NPAHs in the five fractions were calculated. The result is illustrated in Fig. 2. In fraction 4, 52.7% of the mutagenicity was attributed to four NPAHs (1-NP and 1,3-, 1,6- and 1,8-DNPs), while the contribution of the other NPAHs was very small (less than 0.5%). Although, 32.9% of the total mutagenicity of the five fractions was attributed to the above eight NPAHs, there was no NPAH in fraction 5. From the elution order, the mutagen in this fraction seemed to be more polar than NPAHs. Studies to identify this mutagenic compound are currently being conducted.

**Effects of Coexisting Compounds** To study the effects of coexisting compounds on the mutagens, fraction 4, which contained NPAHs, was used as a test solution. The direct-acting mutagenicity was assayed before and after the addition of each fraction to fraction 4. The result is illustrated in Fig. 3. Suppression of 28, 14 and 24% was

![Graphical representation](image)

**Table 1. Concentrations of NPAHs**

<table>
<thead>
<tr>
<th>NPAH</th>
<th>Fr. 1</th>
<th>Fr. 2</th>
<th>Fr. 3</th>
<th>Fr. 4</th>
<th>Fr. 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-NF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>1-NP</td>
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<td>6.48</td>
<td>ND</td>
<td>ND</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>4-NP</td>
<td>ND</td>
<td>0.02</td>
<td>0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
<td>0.10</td>
<td>ND</td>
</tr>
<tr>
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<td>ND</td>
<td>ND</td>
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<td>6-NPPer</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>1,3-DNP</td>
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<td>ND</td>
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<tr>
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<td>ND</td>
<td>0.07</td>
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<tr>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected. Unit, pmol mg\(^{-1}\).
Table 2. Concentrations of PAHs

<table>
<thead>
<tr>
<th>PAH</th>
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<th>Fr. 3</th>
<th>Fr. 4</th>
<th>Fr. 5</th>
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<td>Fluoranthene</td>
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<tr>
<td>Pyrene</td>
<td>ND</td>
<td>6.59</td>
<td>31.6</td>
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<td>Benz[a]anthracene</td>
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<td>0.54</td>
<td>2.28</td>
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<td>ND</td>
</tr>
<tr>
<td>Chrysene</td>
<td>ND</td>
<td>1.66</td>
<td>6.84</td>
<td>ND</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Benzo[a]pyrene</td>
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<td>0.36</td>
<td>1.26</td>
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<tr>
<td>Benzo[ghi]perylene</td>
<td>ND</td>
<td>0.48</td>
<td>2.22</td>
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<td>ND</td>
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<tr>
<td>Benzo[ghi]perylene</td>
<td>ND</td>
<td>0.95</td>
<td>ND</td>
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</tbody>
</table>

ND, not detected. Unit, pmol/mg.

eluted in only fractions 2 and 3, as shown in Table 2. When 1-NP and 1,8-DNP were used as test mutagens, their activity was suppressed by the addition of pyrene or benzo[a]pyrene.

From these results, PAHs are considered to be possible suppressors of the direct-acting mutagenicity of NPAHs in the crude DEP extract. Attempts to identify the suppressors in fraction 1 are now being made.

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References and Notes