Chem. Pharm. Bull. **45**(11) 1820—1822 (1997)

Vol. 45, No. 11

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

Kazuichi HAYAKAWA,* Akio NAKAMURA, Noriko TERAI, Ryoichi KIZU, and Kyoko ANDO

Faculty of Pharmaceutical Sciences, Kanazawa University, 13–1 Takara-machi, Kanazawa 920, Japan.

Received June 12 , 1997; accepted July 28, 1997

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%). Nitropolycyclic aromatic hydrocarbons (NPAHs) were determined by high-performance liquid chromatography with chemiluminescence detection. They were found mainly in fraction 4, although one NPAH 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 NPAHs. The contribution of 1-nitropyrene and 1,3-, 1,6- and 1,8-dinitropyrenes was great and that of the other NPAHs 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 NPAHs.

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 nitropolycyclic aromatic hydrocarbons (NPAHs). Many studies have been done on PAHs in DEP as well as in airborne particulates. However, studies on the behavior and mutagenic contribution of NPAHs 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 NPAHs has been reported.[1–5] Of the NPAHs, 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 detection[7,8] 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 NPAHs was highest in the finest particulate fraction ($<1.1\,\mu$m) 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 NPAHs were identified and their mutagenic contribution was calculated. Also, the effects of coexisting compounds on the direct-acting mutagenicity of NPAHs 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-Nitrobenzo[*a*]pyrene (6-NBaP) and 3-nitroperylene (3-NPer) were purchased from Chemsyn Science Laboratories (Lenexa, 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-nitrochrysene (6-NC) were kindly provided by Dr. N. Sera of the Fukuoka Institute of Health and Environmental Science (Fukuoka, Japan). Fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*f*]fluoranthene, benzo[*a*]pyrene and benzo[*ghi*]perylene were purchased from Kanto Chemical (Tokyo, Japan). Nutrient broth (No. 2), glucose and agarose were purchased from Oxoid (Hampshire, UK), Wako Pure Chemical (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Dimethylsufoxide (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 l min^{-1} and the filter was changed every 15 min. After the filters were dried in a desiccator for 2 d and weighed, they were stored in a freezer ($-20\,^{\circ}$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 redissolved 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 redissolved in 4 ml acetonitrile for the determination of NPAHs and PAHs, respectively, and one in 2 ml of DMSO for the mutagenicity assay.

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

---

* To whom correspondence should be addressed.

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 5C18MS (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 201TP 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 *S. typhimurium* YG 1024 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.

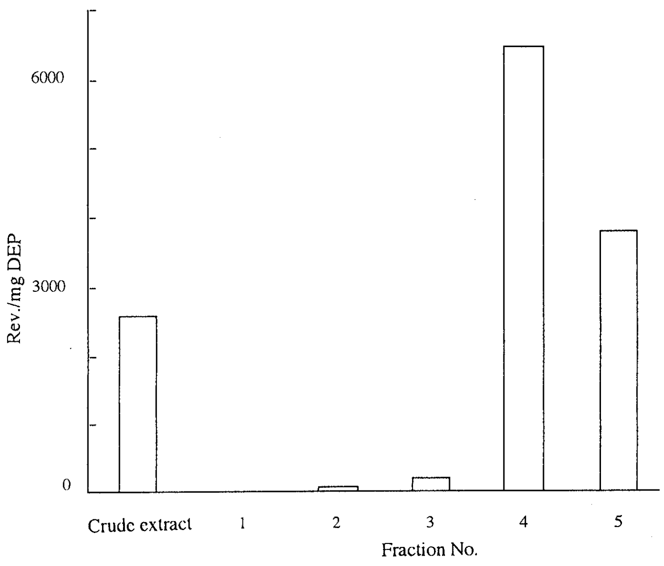

Fig. 1. Direct-Acting Mutagenicity of the Benzene–Ethanol Extract from DEP (Crude Extract) and Five Fractions of DEP Obtained by Silica-Gel Chromatography

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.[9] Recently, 2-NF, 2-, 4-NPs, 6-NC, 6-NBaP and 3-NPer were also determined.[16] 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-NPer, which was eluted in fraction 3. The concentration of 1-NP was about one order of magnitude higher than that of 6-NPer 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-NPer, 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

Table 1. Concentrations of NPAHs

NPAH	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5
2-NF	ND	ND	ND	ND	ND
1-NP	ND	ND	0.01	6.48	ND
2-NP	ND	ND	ND	ND	ND
4-NP	ND	ND	0.02	0.02	ND
6-NC	ND	ND	ND	0.10	ND
6-NBaP	ND	ND	ND	0.30	ND
6-NPer	ND	ND	0.64	ND	ND
1,3-DNP	ND	ND	ND	0.17	ND
1,6-DNP	ND	ND	ND	0.04	ND
1,6-NDP	ND	ND	ND	0.07	ND

ND, not detected. Unit, pmol mg^{-1}.

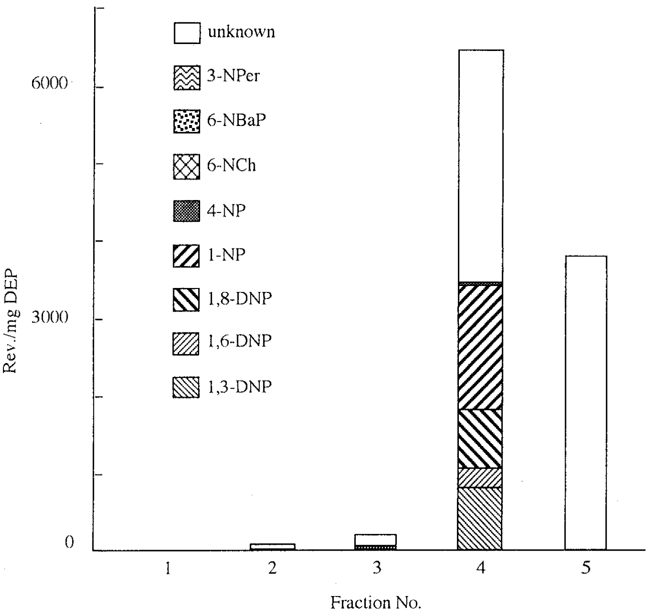

Fig. 2. Calculated Contributions of NPAHs to the Direct-Acting Mutagenicity of the Five Fractions in Fig. 1

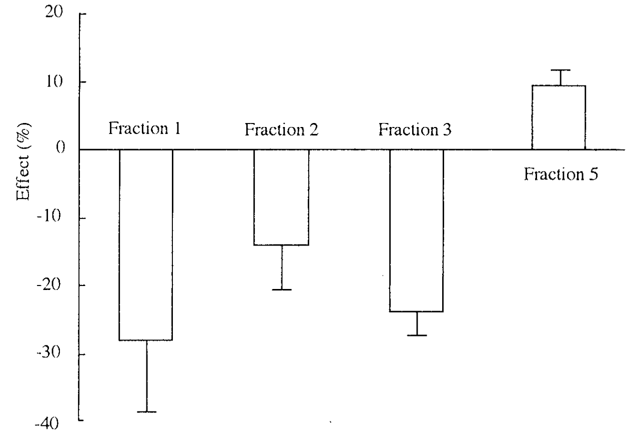

Fig. 3. Effects of Fractions 1, 2, 3 and 5 on the Direct-Acting Mutagenicity of Fraction 4

The direct-acting mutagenicity of fraction 4 was assayed before and after the addition of an equal volume of the test fraction. Values are expressed as a percentage of the values of fraction 4 obtained before addition of the test fraction. Each column and bar represent the mean and SD of three experiments.

observed for fractions 1, 2 and 3, respectively. Moreover, the activity of 1-NP with the crude extract was lower than that of 1-NP without it.

Fractions 1, 2 and 3 were eluted with *n*-hexane and *n*-hexane–dichloromethane (3 : 1, v/v) which were less polar than the dichloromethane used for fraction 4. Although many compounds might be eluted in these fractions, PAHs such as benzo[*a*]pyrene are considered suppressors of NPAHs.[17,18] Stacking interactions between the aromatic rings of PAH and NPAH and suppression of nitroreductase activity were considered as possible mechanisms. Therefore, the concentration of the eight PAHs having 4 to 6 rings was determined in the five fractions. All PAHs were separated from NPAHs and 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.

Table 2. Concentrations of PAHs

PAH	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5
Fluoranthene	ND	2.69	15.9	ND	ND
Pyrene	ND	6.59	31.6	ND	ND
Benz[*a*]anthracene	ND	0.54	2.28	ND	ND
Chrysene	ND	1.66	6.84	ND	ND
Benzo[*b*]fluoranthene	ND	0.81	3.78	ND	ND
Benzo[*f*]fluoranthene	ND	0.36	1.26	ND	ND
Benzo[*a*]pyrene	ND	0.48	2.22	ND	ND
Benzo[*ghi*]perylene	ND	0.95	ND	ND	ND

ND, not detected. Unit, pmol mg^{-1}.

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.

Acknowledgements We wish to thank Mr. Fumio Kano of the Tokyo Metropolitan Research Laboratory of Public Health for providing the *S. typhimurium* YG1024 strain.

References and Notes

1) Gibson T. L., *Mutat. Res.*, **122**, 115—121 (1983).
2) Schuetzle D., *Environ. Health Perspect.*, **47**, 65—80 (1983).
3) Siak J., Chan T. L., Gibson T. L., Wolff G. L., *Atmos. Environ.*, **19**, 369—376 (1985).
4) Arey J., Zielinska B., Harger W. P., Atkinson R., Winer A. M., *Mutat. Res.*, **207**, 45—51 (1988).
5) Strandell M. S., Zakrisson S., Alsberg T., Westerholm R., Winquest L., Rannug U., *Environ. Health Perspect.*, **102**, Sup. 4, 85—92 (1994).
6) Rosenkranz H. S., Mermelstein R., *Mutat. Res.*, **114**, 217—267 (1983).
7) Hayakawa K., Kitamura R., Butoh M., Imaizumi N., Miyazaki M., *Anal. Sci.*, **7**, 573—577 (1991).
8) Hayakawa K., Butoh M., Miyazaki M., *Anal. Chem. Acta*, **266**, 251—256 (1992).
9) Hayakawa K., Butoh M., Hirabayashi U., Miyazaki M., *Jpn. J. Toxicol. Environ. Health*, **40**, 20—25 (1994).
10) Hayakawa K., Murahashi T., Butoh M., Miyazaki M., *Environ. Sci. Technol.*, **29**, 928—932 (1995).
11) Murahashi T., Miyazaki M., Kakizawa R., Yamagishi Y, Kitamura M., Hayakawa K., *Jpn. J. Toxicol. Environ. Health*, **41**, 328—333 (1995).
12) Hayakawa K., Kawaguchi Y., Murahashi T., Miyazaki M., *Mutat. Res.*, **348**, 57—61(1995).
13) Murahashi T., Hayakawa K., *Anal. Chim. Acta*, **343**, 251—257 (1997).
14) Kado N. Y., Langley D., Eisenstadt E., *Mutat. Res.*, **121**, 25—32 (1983).
15) Watanabe M., Ishidate M., Jr., Nohmi T., *Mutat. Res.*, **234**, 337—348 (1990).
16) Murahashi T., Ando K., Kizu R., Kakimoto H., Hayakawa K., submitted.
17) Matsushita H., Tanabe K., Endo O., Takahashi K., Murata M., Lee H., Hayano S., *J. Jpn. Soc. Air. Pollut.*, **21**, 211—215 (1986).
18) Lee H., Cheng S. H., Liu T. Y., *Environ. Mol. Mutagen*, **24**, 229—234 (1994).