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Degradation by a Pyrene-Assimilating Bacterium of Polycyclic Aromatic Hydrocarbons in Heavy Oil Washed Ashore at Mikuni Coast

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ABSTRACT

Benz(a)anthracene (BaA), pyrene, benzo(a)pyrene (BaP), and benzo(ghi)perylene in the heavy oil that washed ashore (WA-heavy oil), which spilled from the wrecked Russian oil tanker Nakhodka, disappeared at levels of 99%, 92%, 60%, and 33%, respectively, by *Mycobacterium* sp. H2-5 at 30℃ for 20 days, which was isolated as a pyrene-assimilating bacterium in 1994. Similarly, these high-molecular-weight polycyclic aromatic hydrocarbones (HPAHs) in commercial heavy oil (class C) disappeared at levels of 92%, 99%, 60%, and 10%, respectively, by the bacterium under the same condition. There were no great differences in the disappearances of HPAHs between the two heavy oil samples by strain H2-5 (Mineki *et al.*, 1998). Moreover, the change of mutagenicity during degradation of HPAHs in WA-heavy oil, by strain H2-5 was investigated. The mutagenicity of the benzene extract from the solution, which was treated with the strain H2-5 for 60 days, increased dose-dependently only when incubated with S9mix, as did the mutagenicity of extract from the control. The mutagenicities of the aqueous layers in 0, 15, 30, 45 and 60 days' treatment, were not significantly detected, and they were independent of S9mix. Since the treatment of WA-heavy oil by H2-5 did not increase the level of mutagenicity of it, it did not seem to have undesirable side effects (Mineki *et al.*, 2003).

1. Degradation by a Pyrene-Assimilating Bacterium of Polycyclic Aromatic Hydrocarbons

Energy consumption is considered to be increasing with the development of industries today, and the greenhouse effect by carbon dioxide, nitrous oxide, methane, and so on, has been a serious worldwide problem. Transit of crude oil and its related oils as the main energy source of industries has been properly increased, yet pollution of coasts with spilled oil by accidents of oil tankers has repeatedly occurred. Such pollution is considered to damage the ecosystem of the site, and consume a long-term period to recover it. That is to say, polycyclic aromatic hydrocarbons (PAHs), which are natural constituents of fossil fuels (Matsushita, 1972; Attias *et al.*, 1995), are a class of potentially carcinogenic chemicals in the environment in addition to mycotoxins and nitrosoamines. The degradation of HPAHs (Heitkamp *et al.*, 1988; Mueller *et al.*, 1990), containing four or more benzene rings, by microorganisms is generally more difficult than that of lower-molecular-weight PAHs (LPAHs), such as naphthalene, anthracene, and phenanthrene (McKenna, 1976; Gibson and Sabramanian, 1984). Therefore, remediation of sites contaminated with HPAHs is very crucial as a countermeasure against these environmental carcinogens. Although degradation of *n*-alkanes and LPAHs by a variety of bacteria has been demonstrated, investigation of biodegradation of HPAHs remains scarce. *Mycobacterium* sp. H2-5, which was isolated as a pyrene-assimilating bacterium in 1994 (Mineki *et al.*, 1994), degraded HPAHs in extract from airborne particles and in extract (tarry matter) from soil (Mineki *et al.*, 1996).

In this study, we describe the disappearance of several HPAHs in heavy oil that washed ashore at Mikuni-cho in Fukui Prefecture, which spilled from the wrecked Russian oil tanker Nakhodka on Jan. 2, 1997, by *Mycobacterium* sp. H2-5.

1.1 MATERIALS AND METHODS

Materials

Heavy oil that washed ashore was collected at Echizenmatsushima, Mikuni-cho in Fukui Prefecture on Jan. 16, 1997. As standard for PAHs, pyrene, benz[a]anthracene

(BaA), and benzo[a)]pyrene (BaP) were purchased from Wako Pure Chem. Ind., Ltd., and benzo[ghi]perylene (BghiP) was purchased from Aldrich Chem. Co. Inc. Fluorometry grade reagents and HPLC grade reagents were used for extraction of HPAHs and for HPLC analyses, respectively, without further purification. Other reagents were of GR-grade and were used as received.

Media

For a complete medium, TSB medium was used; it contained 30 g of Tryptic soy broth (Difco) per 1 liter of distilled water; pH 7.0. The mineral salt medium (MM) used for enrichment and culturing of pyrene-degrading bacteria contained, per liter, 1 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of KH_2PO_4, 1.5 g of $(NH_4)_2SO_4$, 1 g of NH_4Cl, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 0.015 g of $CaCl_2 \cdot 2H_2O$, 0.01 g of $FeCl_3 \cdot 6H_2O$; the pH was adjusted to 7.0. As a carbon source, 0.02 ml of pyrene solution (20 mg/ml dimethylformamide (DMFA)) was added to 10 ml of MM (pyrene-MM) in an L-tube (ϕ 18 mm x 120 mm).

Cultivation of bacteria

After strain H2-5 was cultured in an L-tube containing 10 ml of TSB medium on a reciprocal shaker (100 strokes/min) at 30°C for one month, 5 ml of the culture was inoculated to 250 ml of MM containing 2.5 ml of the pyrene solution (DMFA) in a 500-ml of Erlenmeyer flask, and then incubated on a rotary shaker (160 rpm) at 30°C. Growth of strain H2-5 was evaluated by measuring the protein of filter cake of the culture broth by the method of Lowry *et al.* (1951). The culture was harvested at the stationary phase (after 30 days) by centrifugation and the pellet was washed with MM, and used in subsequent investigations.

Electron microscopy of strain H2-5

For scanning electron microscopy, a clump of cells was fixed for 30 min in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) on a Sempore filter (JEOL Ltd.). It was then lyophilized in *t*-butanol, and sputter coated with gold. The colony was observed in a JEOL JSM-5510LV scanning microscope.

Degradation of PAHs in the heavy oil

After the heavy oil solution (0.1 mg/0.3 ml benzene) was added to the L-tube, which held 30 glass beads (ϕ 30 mm), it was shaken gently, and then the solvent was evaporated under reduced pressure. The cell solution (0.8 mg dry-cell weight/5 ml)

and 5 ml of MM were added to the L-tube, followed by incubation on a reciprocal shaker (100 strokes/min) at 30°C.

Extraction of PAHs

The extraction of HPAHs was done by modifying the method of Takagi *et al.* (1986). The culture was extracted with 20 ml of cyclohexane in a screw-topped test tube by shaking vigorously, and the organic layer was collected and concentrated down to 1 ml under nitrogen stream. HPAHs in the concentrate were extracted with 0.5 ml of dimethyl sulfoxide and analyzed by HPLC.

Analyses of PAHs

The concentrations of HPAHs were measured spectrophotometrically (Tanabe *et al.*, 1987). Namely, the intensity of fluorescence of each sample was measured by a Hitachi 850 fluorescence spectrophotometer, to determine the relative concentration with a control (pyrene: Excitation, 339 nm, Emission, 373 nm; BaA: 292 nm, 412 nm; BaP: 370 nm, 406 nm; BghiP: 370 nm, 406 nm). HPAHs extracted from the heavy oils were separated into individual HPAHs by Shimadzu LC-6A HPLC using ODS-60-5 double columns (4.6 x 30 mm for concentration, and 4.6 x 250 mm for separation, Tokyo Chemical Industry, Tokyo, Japan). After the application of the sample solution, HPAHs were concentrated by a first column with a mixed solution of acetonitrile (AN)/H_2O=1:1 for 5 min, and then they were separated by a second column with AN/H_2O=4:1.

1.2 RESULTS AND DISCUSSION

Electron microscopy of strain H2-5

Fig. 1 shows SEM of *Mycobacterium* sp. H2-5 cultured on pyrene and MM agar slant at 30°C for 30 days. Cells were rod shaped approximately 0.5 μm in width and 1-2 μm in length.

Disappearance of HPAHs in the commercial heavy oil (Class C)

The commercial heavy oils (Class C) have a variety of PAH compositions, and these differences depend on the district. The heavy oil samples used in this study mainly contained pyrene, BaA, BaP, and BghiP as HPAHs (**Table 1**). As shown in **Fig. 2**(A), strain H2-5 strongly degraded pyrene, and it almost disappeared (99% decrease against the control) after incubation for 5 days. BaA and BaP also

levels of 92% and 60%, respectively, by strain H2-5 after incubation for 20 days. On the other hand, BghiP hardly disappeared under the same condition. This result may be due to the structural difference that BghiP has more aromatic rings (6 rings) as compared with the former 3 HPAHs, which have 4 or 5 rings.

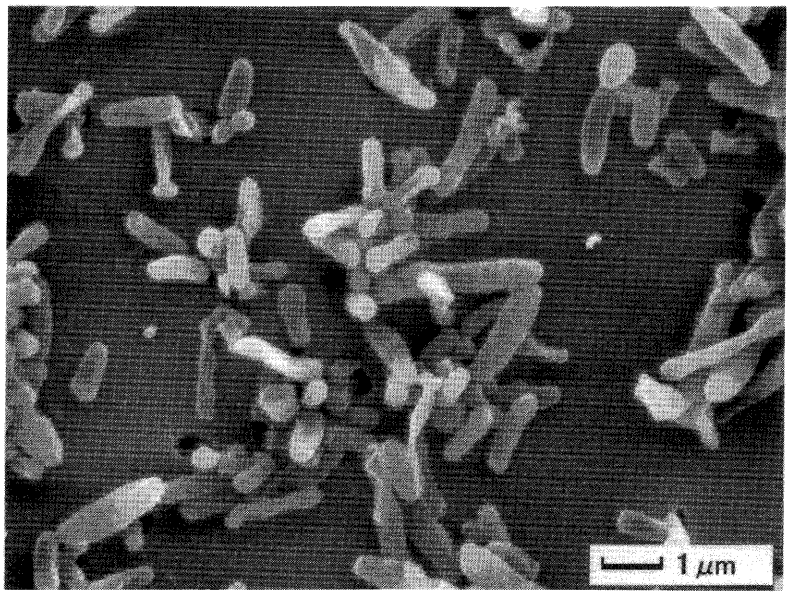

Fig. 1 Scanning electron micrograph of strain H2-5.

Table 1 HPAH contents in two heavy oil samples.

PAH	Com-HO[a]	HO-WA[b]
Pyrene	63.8	12.6
BaA	9.6	20.2
BaP	6.3	4.0
BghiP	25.6	26.2

ng/0.1 mg oil/L-tube.
[a] Commercial heavy oil (Class C).
[b] Heavy oil that washed ashore.

Disappearance of HPAHs in the heavy oil that drifted ashore

The heavy oil that drifted ashore contained the same HPAHs as the commercial heavy oil, but two heavy oils differed much on the pyrene and BaA contents (**Table 1**). As shown in **Fig. 2**(B), pyrene disappeared rapidly (92% after 20 days) as was seen in the commmercial heavy oil. Although BaA did not disappear with 10 days' treatment, subsequently it was abruptly degraded and decreased to be undetected after 15 days. This phenomenon may be due to the high content of BaA in the heavy oil that washed

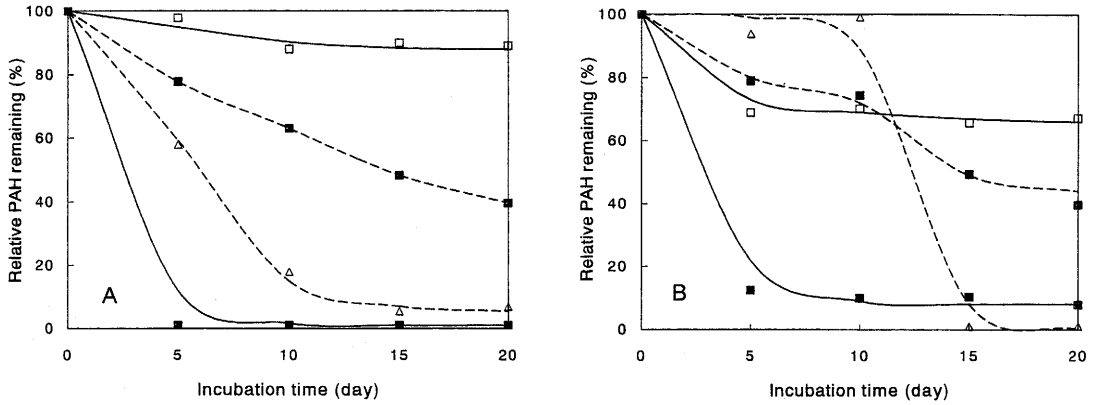

Fig. 2 Disappearance of HPAHs in two heavy oil samples by strain H2-5.
A: Commercial heavy oil (Class C); **B**: Heavy oil that washed ashore;
(—■—) pyrene; (---△---) BaA; (---■---) BaP; (—□—) BghiP.

ashore. Disappearances of BaPs in both heavy oils were almost the same degree (60% after 20 days). BghiP was not well degraded (33%) even after 20 days, but this value was 3-fold higher than that of commercial heavy oil. The cause of this difference is not clear at present. Further investigations are necessary including the effect of interaction of PAHs. Negative effects of sea water and photochemical products by drifting on the degradation of HPAHs in the heavy oil by strain H2-5 had been feared, but there was no great negative effect in those results. Therefore, no harm was done by the drifting of heavy oil for two weeks, to the activity of the bacterium. But a possibility that HPAHs change to compounds that are more resistant to the bacterial degradation, or that inhibitory compounds are produced after longer-term drifting, cannot be neglected. In our previous work (Mineki *et al.*, 1994), BaA did not disappear individually as much as in the results of this study; therfore, a certain compound in the heavy oil thought to facilitate the degradation of BaA, i. e., cooxidation (McKenna, 1976) with it, may be present.

In our other study (Goto *et al.*, 1997), mutagenicity of the extract by methanol from the heavy oil that drifted ashore was 230,000 rev/g, which was 3- to 4-fold higher than the values for the extracts from the soil samples at urban areas in Tokyo in 1984 and 1996. Since a respectable amount of the heavy oil was thought to filter into the sand of the coast, its effect on the ecosystem there must be watched.

2. Effect on mutagenicity of degradation by *Mycobacterium* sp. of polycyclicaromatic hydrocarbons in heavy oil washed ashore

In addition to volatile aromatic compounds, heavy oil contains HPAHs which have mutagenicity, carcinogenicity and endocrine-disruptive activity. HPAHs are hardly volatile and scarcely degraded by microorganisms (Heitkamp *et al.*, 1988). Therefore, HPAHs are of concern as to them remaining behind in the environment and causing damage to the ecological system, and further even to humans. In **part 1**, we stated about the microbial degradation of HPAHs in heavy oil that spilled from a wrecked oil tanker and washed ashore. It is essential, however, to avoid causing more serious contamination by bioaugmentation. Namely, metabolites occurred by the action of microbes from contaminants may increase the level of mutagenicity more than the original level. Although the mutagenicity must naturally change depending on the actions of coexisting microorganisms, we brought to a focus on the change of mutagenicity, in this study, during degradation of HPAHs in WA-heavy oil by the *Mycobacterium* sp. H2-5.

2.1 MATERIALS AND METHODS

Cultivation of bacteria

Mycobacterium sp. H2-5 was precultivated with 10 ml of MM supplemented with 2.0 mg of pyrene as a carbon source in an L-tube at 30°C for 30 days with reciprocal shaking at 100 rpm. Then, 4 ml of preculture was inoculated to 200 ml of MM with addition of 40 mg of pyrene in a 500-ml Erlenmeyer flask and it was cultured at 30°C for 30 days with rotary shaking at 160 rpm. The cells were harvested by centrifugation (15,000 x g, 15 min), washed twice with distilled water, and once with MM, and they were resuspended in MM. Dry-cell weight was calculated by deducing the weight of control (10 ml of MM) from the weight of cell suspension (10 ml) after keeping them at 100°C for 20 hr.

Degradation of PAHs in the heavy oil and extraction by benzene

Heavy oil that leaked from a wrecked Russian tanker, the Nakhodka, and washed ashore was collected at Echizenmatsushima, Mikuni-cho in Fukui Prefecture on Jan. 16,

1997. WA-heavy oil was stored at -20°C until use.

Heavy oil was dissolved in benzene (2.0 mg/1.2 ml) and poured into a sterilized L-tube holding 30 glass beads (3 mmϕ). After gently shaking it, the solvent was evaporated. H2-5 cell suspension (2.6 mg dry-cell weight/10 ml MM) was added to the extract, followed by incubation on a reciprocal shaker (100 rpm) at 30°C for 60 days. Ten ml of MM was added instead of the cell suspension, for a control. Remaining HPAHs were extracted from the reaction mixture with 10 ml of benzene and this extraction was repeated eight times. Each benzene layer was combined and filtered (Dimex, pore size: 0.2 μm, Millipore Co., Bedford, USA). On the other hand, an aqueous layer was dried at room temperature and dissolved with methanol, following by filtering with Dimex. Both filtrates from the benzene layer and the aqueous layer were individually evaporated and dried under a nitrogen stream. Each extract from the benzene layer and the aqueous layer was dissolved with 2 ml (equivalent to WA-heavy oil of 1.0 mg/ml, abbr. 1.0 mg heavy oil eq./ml) of dimethyl sulfoxide (DMSO) and 1 ml of sterilized distilled water, respectively, and then their mutagenicities were measured.

Measurement of mutagenicity

Mutagenicities of the extracts from the reaction mixture were measured employing a pre-incubation method (Yahagi *et al.*, 1997) which is the modified Ames test (Ames *et al.*, 1975). A cell suspension (100 μl) of *Salmonella typhimrium* TA100 and 100 μl of the extract solution were mixed with 500 μl of S9mix (Oriental Yeast Co. Ltd., Tokyo) or without S9mix (with an equivolume of sterilized distilled water).

Analyses of HPAHs

Each concentration of pyrene, BaA, BaP and BghiP in the extract (DMSO solution) by benzene, was analyzed by HPLC using a Shimadzu LC-6A, ODS-60-5 double columns (4.6 x 30 mm and 4.6 x 250 mm, Tokyo Chemical Industry, Co. Ltd., Tokyo) and a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan).

2.2 RESULTS AND DISCUSSION

In a previous paper (Goto *et al.*, 1997), we reported that mutagenicities of methanol extracts from WA-heavy oil were remarkably stimulated when S9mix was added to the

reaction mixture. However, metabolites of the heavy oil by the strain H2-5 may have mutagenicities. Therefore, mutagenicities of the treated solutions with H2-5 were measured under both presence or absence of S9mix. **Fig. 3** shows mutagenicity of the benzene extract from the solution treated with the strain H2-5 for 60 days. The mutagenicity increased dose-dependently only when incubated with S9mix, as did the mutagenicity of extract from the control. The mutagenicities of benzen extracts in 0, 15, 30 and 45 days' treatments were much the same as 60 days' treatment. Therefore, it was found that benzene extracts from WA-heavy oil and from the solutions treated with H2-5, predominantly contained promutagens, rather than direct mutagens, as did in methanol extracts (Goto *et al.*, 1997). Mutagenicity of benzene extract during the

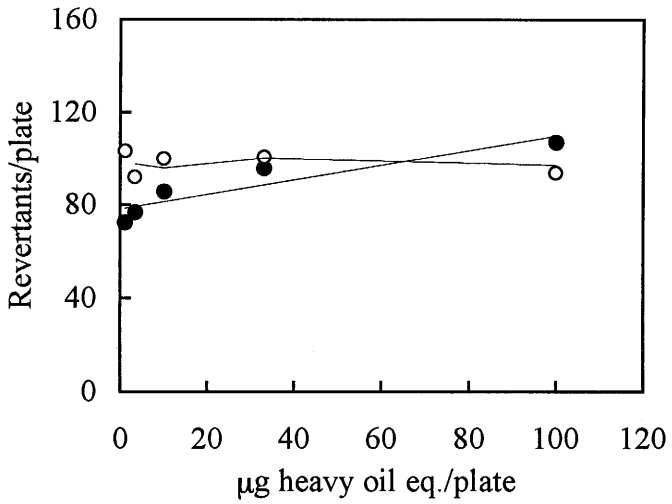

Fig. 3 Mutagenicity of the benzene extract from the reaction mixture by the preincubation assay employing *S. typhimurium* TA100. Symbols: ●, +S9mix; ○, -S9mix.

treatment at the concentration of 100 μg heavy oil eq./plate hardly decreased and it was not much different from that of the control, as shown in **Fig. 4**. Degradation of each PAH in WA-heavy oil by H2-5 is shown in **Fig. 5**. BaP (initial concentration: 29 μg/g-oil) and BghiP (initial concentration: 113 μg/g-oil) were reduced to 60% and 70%, respectively, by the incubation for 30 days. Quantifications of pyrene and BaA after 15 and more days' incubation were obstructed by impurities in the benzene extracts. However, these HPAHs in cyclohexane extracts had been confirmed to degrade exceedingly easier than BaP and BghiP by H2-5 in our previous studies (Mineki *et al.*, 1996, 1998). Most of pyrene and BaA, therefore, considered disappearing in the

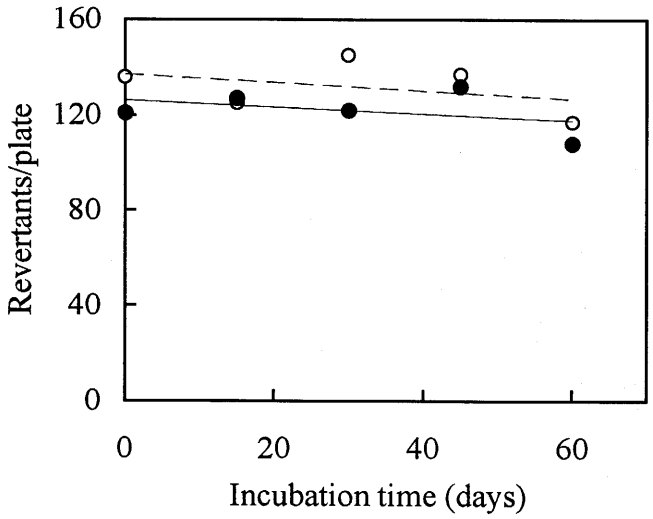

Fig. 4 Mutagenicity of the benzene extract during the treatment by H2-5. Symbols: ●, treated by H2-5; ○, control.

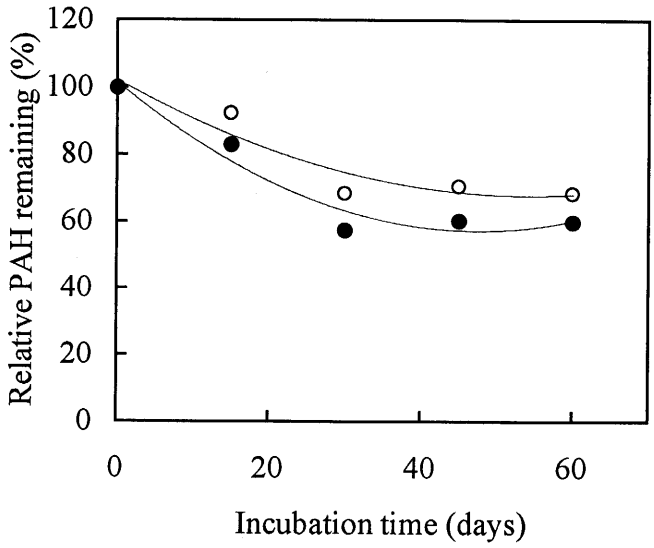

Fig. 5 Degradation of PAHs in WA-heavy oil by H2-5. Symbols: ●, BaP; ○, BghiP.

reaction mixture after 60 days. From these results, it was found that the mutagenicity 15 and more days' incubation were obstructed by impurities in the benzene extracts. However, these HPAHs in cyclohexane extracts had been confirmed to degrade exceedingly easier than BaP and BghiP by H2-5 in our previous studies (Mineki *et al.*, 1996, 1998). Most of pyrene and BaA, therefore, considered disappearing in the reaction mixture after 60 days. From these results, it was found that the mutagenicity

of the reaction mixture hardly decreased, in spite of reducing the level of strong mutagen as BaP to 60% of the initial level.

The mutagenicities of the aqueous layers in 0, 15, 30, 45 and 60 days' treatment, were not significantly detected, and they were independent of S9mix. Namely, water-soluble products that occurred as a result of the treatment of WA-heavy oil with H2-5 scarcely have mutagenicities for TA100.

Mutagenic activity of benzene extract from WA-heavy oil was calculated to 448,000 revertants/g-oil (rev/g), and that of the extract from soil from an urban area in Tokyo in 1984 was 56,000 rev/g (Nishimura *et al.*, 1984), that was 12.5% of the level for WA-heavy oil. On the other hand, BaP contents of WA-heavy oil and the extract from soil in an urban area in Tokyo in 1984 were 29 μg/g-oil and 44.4 μg/g (geometric mean: 31.6 μg/g) (Nishimura *et al.*, 1984), respectively, i.e., BaP level in the extract from soil was 1.5-fold higher than that in WA-heavy oil. Comparing the BaP content and mutagenic activity of WA-heavy oil with those of extract of soil in Tokyo, it was predicted that WA-heavy oil contained other strong mutagens besides BaP. Since the treatment of WA-heavy oil by H2-5 did not increase the level of mutagenicity of it, it did not seem to have undesirable side effects.

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