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IDENTIFICATION OF CARCINOGENIC TRP-P-2 AND -1 IN THE EFFLUENT FROM A SEWAGE TREATMENT PLANT

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It has been identified that the origin of the heterocyclic amines, a group of suspected human carcinogens is high-temperature heating of proteinaceous materials, e.g., food and plants (tabacco-leaves and woods). The heterocyclic amines have been found in human excretions and rain water. We extracted two of these heterocyclic amines, 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) in the water of a sewage teatment plant in Japan by HPLC analysis. The concentration of Trp-P-2 and -1 were estimated at about 10^{-2} μ g/L, each. The presence of these compounds shows the possible widespread pollution of surface waters with this class of carcinogens.

Key Words : *heterocyclic amines, carcinogen, sewage effluent, Trp-P-2, Trp-P-1, umu test, mass spectrometric analysis, high performance liquid chromatograph*

1. INTRODUCTION

Sources of drinking water are being increasingly polluted by chemicals. For example, the issues of source-water pollution by pesticides and organic halides have been prominent. One reason for the problems associated with the supply of source-water in Japan, where the available water mass is small, is the necessity of the use of waste water, after its treatment, to fulfill the demand. Thus, processed water discharged from sewage treatment plants is often mixed with the sources of drinking water in urban areas of Japan

We are concerned about pollution of the source waters by genotoxic materials. It has been reported that strongly mutagenic heterocyclic amines are formed by cooking meat and fish, and that these mutagens come out of humans into urine and feces[1),11),12),14)]. Also, tabacco burning and forest-fire seem to produce these mutagens[15)]. Heterocyclic amines are rodent carcinogens and are regarded as possible or probable human carcinogens. These compounds are found in rain water[15)] and in water-samples of a human waste-treatment plant, a fact suggesting that the heterocyclic amines are ubiquitously present in the environment. In the surveyed areas, rivers have been reported to be polluted with mutagens, but the nature of these mutagens is mostly unknown.

In the work to be described below, we explored the possibility of mutagen pollution in the water samples of a municipal sewage treatment plant. The effluent of this plant is discharged into the river, which is the source of drinking water of the million people in the downstream area. In this sewage plant, sewage from homes, including drains in toilets, and waste water from various industries are collected and treated. In this study, we have attemped to isolate and identify the heterocyclic amines in the effluent of a sewage treatment plant.

2. MATERIALS AND METHODS

(1)The sample

In the sewage treatment plant studied this time, about 70 % of the particles in the influent sewage at the plant are removed in the primary sedimentation tank. The residual organic materials present in the supernatant fluid are either decomposed by or transformed in the activated

sludge composed of micro-organisms. The grown sludge is separated from the liquid in the secondary sedimentation tank. After a disinfection by chlorination, the resulting supernatant is discharged into the river. Samples were collected in December 1995 and May 1996 from the effluent of the secondary sedimentation tank.

(2)Extraction

Blue-rayon (Funakoshi Pharmaceutical Co. Ltd. ,Tokyo) which is rayon bearing covalently bound trisulfo-copper-phthalocyanine as a ligand for polycyclic aromatic compounds, was used to adsorb the mutagenic substances contained in the sample. Each sample was first filtered with a 1 μm glassfiber filter. The sample was passed through a blue-rayon column (6mm $\phi \times 200$ mm , 1.0 gr.) at a flow rate of 10 mL/min. The sample size was 40 litres per column. The column was washed with distilled water, and the moisture in each column was removed by air flow. The substances adsorbed on the blue-rayon were eluted with methanol-25% ammonia (100 mL, 50:1, v/v) at a flow rate of 2 mL/min. The eluate was collected and evaporated to dryness under reduced pressure in a rotary evaporator at 35 ℃. The residue thus obtained was dissolved in methanol for the genotoxicity assay and the HPLC separation process.

(3)HPLC Analysis of Heterocyclic Amines and their Mass Spectrometric Analysis

Reversed-phase HPLC analysis was performed by using a Shim-pack PREP-ODS column (20 μm particle, 20 mm $\phi \times 250$ mm , Shimadzu, Kyoto). A sample was injected and eluted at a flow rate of 5 mL/min using a mobile phase of methanol-10 mM ammonium acetate (3:2, v/v). The fractions were collected at 2-min intervals throughout the run, monitored by UV absorbance at 265 nm and then each fraction was assayed for genotoxicity. The genotoxic fractions were evaporated to dryness and the residues were dissolved in methanol to submit to second-step HPLC. The second HPLC was performed with an Asahipak ES-502C column (9 μm particle, 7.6 mm $\phi \times 100$ mm , Asahi Chemical Industry Co.,LTD., Tokyo). Elution was performed at a flow rate of 1 mL/min using a mobile phase of acetonitrile-0.1% trifluoroacetic acid (0-10 min gradient: 0-2% acetonitrile/100-98% triflioroacetic acid, then 10-40 min gradients :2-20% acetonitrile/98-80% trifluoroacetic acid). Then, the fractions corresponding to the peaks of Trp-P-1 and Trp-P-2 as determined by a separate HPLC of standard samples were evaporated and dissolved into methanol for the third-step HPLC analysis using a TSKgel ODS120A column (4.6 mm $\phi \times 250$ mm, Tosoh., Tokyo). Elution was performed at a flow rate of 1 mL/min with a mobile phase of acetonitrile - 20 mM phosphoric acid (1:4, v/v). Finally, to identify materials, the fractions corresponding to the position of an authentic sample of Trp-P-2 were evaporated and the residues were dried and dissolved with methanol for direct injection mass spectrometric analysis. The mass spectral analysis was performed in EI mode with a direct insertion probe on a double-focusing JOEL mass spectrometer, with the ionizing current, 0.3 A and the ionizing voltage, 70 eV.

(4)Genotoxicity Assay Umu-test Procedure

a)Tester strains

Salmonella typhimurium strains to be used in the *umu*-test[3] was provided by Dr.Oda of the Osaka Prefectural Institute of Public Health, Japan. *S. typhimurium* NM2009 is a strain having high *O*-acetyltransferaseactivities, and NM2000 lackes the *O*-acetyltransferase activity[13]. A DNA damaging aromatic amine with S9 would show activity in NM2009 but not in NM2000. The genotoxicity is represented by the enzymatic activity found with NM2009 above the background activity with NM2000.

b)Test procedure

An overnight culture of the tester bacterial strain in LB broth (1% bactotrypton, 0.5% NaCl, 0.5% yeast extract, 1000 mL water) was diluted into the TGA medium (1% bactotrypton, 0.5% NaCl, 0.2% glucose, 1000 mL water) which was supplemented with 20 mg/L ampicillin and 10 mg/L chloramphenicol. The mixture was divided into 2.4 mL portions in test tubes, and then, 0.1 mL of a test compounds and S9 mixture with cofactors (0.5 mL) were added to each test tube. After incubation at 37 ℃ for 4 hrs with shaking, β-galactosidase activity in the cell was measured. The *umuC* gene which responds to an SOS stimulus induced by any DNA damage was fused to the *lacZ* gene which codes for β-galactosidase activity. β-Galactosidase activity was determined by the method of Miller[2]. The enzymatic activity was determined colorimetrically using 2-nitrophenyl-β-*D*-galactopyranoside as the substrate. The concentration coefficients is volume-reduction ratio from the original water: for example, 0.1 mL sample concentrated from 100 mL-equivalent

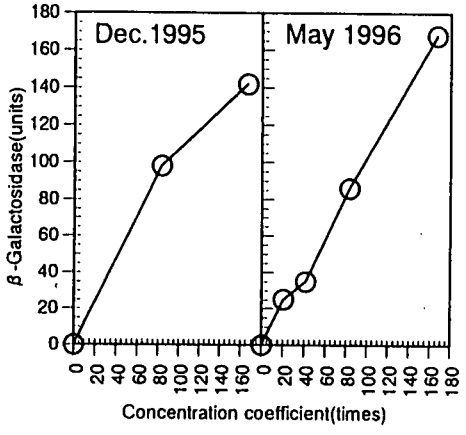

Fig. 1 Dose response of the *umu* test in the presence of *S.typhimrium* NM 2009 and NM2000 strains of the effluent from a municipal sewage treatment plant. (○) represents the difference in each point in the β-galactosidase activity between both the NM2009 strain and the NM2000 strain.

sewage fluid has a coefficient of 1,000.

3. RESULTS

(1)Detection of the genotoxicity of an effluent from a treatment plant treated with blue-rayon

Blue rayon extracts of effluents from the activated sludge treatment tanks at a sewage treatment plant were found to be strongly positive in the *umu*-test. **Fig. 1** shows the dose response curves in the *umu*-test of two samples taken in different dates.

The results showed that genotoxic substances adsorbable to the blue-rayon in the sewage are present in the sewage fluid even after the biological treatment, and that the genotoxic substances are probably polycyclic aromatic amines.

(2)HPLC analysis of heterocyclic amines in the samples

Fig. 2(B) shows the peaks in HPLC on Shim-pack Prep-ODS which seemed to correspond to those of several heterocyclic amines. Authentic heterocyclic amines' chromatograph are shown in the **Fig. 2(A)**. As shown in **Fig. 2(C)**, the strongly genotoxic fractions shown in black were detected , which corresponded to the peaks of MeIQx+IQ, Trp-P-2 and Trp-P-1, respectively (hatched in **Fig. 2(b)**).

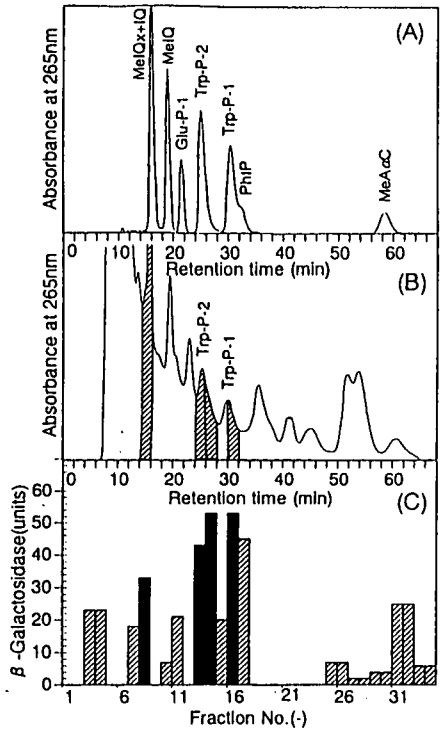

Fig. 2 The HPLC by using Shim-pak Prep-ODS column profiles (A) authentic heterocyclic amines. (B) the blue-rayon extracts of the effluent from a municipal sewage treatment plant. (C) The genotoxicity of the HPLC fractions of the blue-rayon extracts of the effluent from the plant assayed by using *S.typhimrium* named NM 2009 in a presence of the S9mix.

(3)Identification of Trp-P-2 in a genotoxic fraction

The genotoxic fractions Nos.13,14 and 16, which corresponded to the peaks of Trp-P-2 and Trp-P-1, were collected and subjected to further HPLC fractionation with an Asahipak ES-502C column. **Fig. 3(A)** shows the HPLC-UV profile of standard Trp-P-2 and Trp-P-1. The HPLC-UV profile of the sample from the first HPLC is shown in **Fig. 3(B)**. The peaks that corresponded to Trp-P-2 and Trp-P-1 were again collected and fractionated by another HPLC on a TSKgel ODS-120A column. **Fig. 4(A)** shows the HPLC-UV profile of standard Trp-P-2 and Trp-P-1, **Fig. 4(B)** represents the HPLC-UV profile of the sample from the second HPLC (**Fig. 3(B)**). The concentrations of both Trp-P-2 and Trp-P-1 are calculated approximately as 9.1 ng/L and 8.2 ng/L, respectively in the effluent obtained in May 1996.

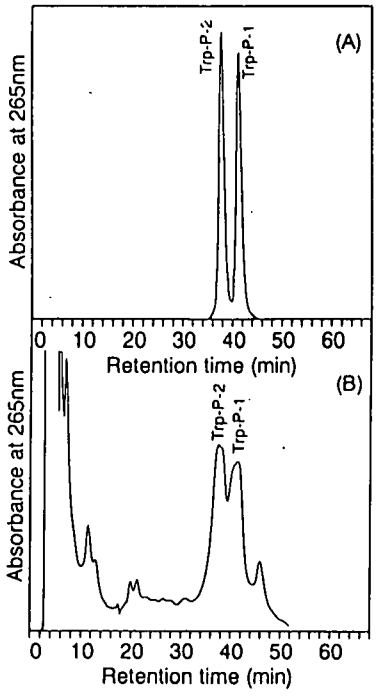

Fig. 3 The HPLC profiles by using Asahipak ES-502C column for the second-step purification (A) authentic Trp-P-1 and Trp-P-2. (B) the blue-rayon extracts of the effluent from the municipal sewage treatment plant.

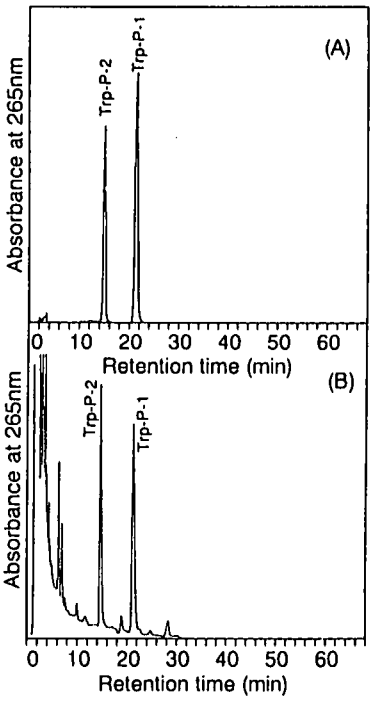

Fig. 4 The HPLC profiles by using a TSKgel ODS-120A column for the third-step purification (A) authentic Trp-P-1 and Trp-P-2. (B) the blue-rayon extracts of the effluent from a municipal sewage treatment plant.

Finally, an identification of the materials was conducted. The fractions purified by these three-step HPLC fractionations were subjected to a direct-injection mass spectrometry analysis. **Fig. 5(A)** shows that the mass spectra of a standard Trp-P-2, which displayed a molecular ion peak at m/z197 and a fragment ion peak at m/z 180. The mass spectra of the fraction obtained in third HPLC, that corresponded to the peak of the Trp-P-2 is shown in **Fig. 5(B)**. Both the molecular in peak at m/z 197 and the fragment peak at m/z 180 were detected. The peak corresponding to Trp-P-1 was not obtained still nonclear in the mass spectra. These results clearly indicate the presence of Trp-P-2 in the effluent.

4. DISCUSSIONS

Researchers on environmental mutagens have found mutagenic activity in water taken from rivers where the sewage treatment plants are located upstream. Their studies have shown that there is strong mutagenic activity in the extracts on the blue-rayon, which adsorbs the polycyclic aromatic hydrocarbons selectively, and they suggest the blame be placed on the effluents from sewage treatment plants as being the culprits in the pollution resources of mutagenic chemicals in rivers[4),5),9)].

In comparison, high mutagenicity in the effluent of a municipal sewage treatment plant could be observed[6)]. This mutagenic activity was thought to be the inducement of aromatic amines, because the test-system NM2009 has rich o-acetyltransferase. The enzyme can acetylize aminoarenes to attack the DNA with P450 enzyme. However, it is too difficult to identify the substance because sewage always contains numerous substances that arise from domestic sewage, human waste and industrial wastewater. Segawa *et al.*[10)] recorded that Trp-P-2 in a municipal sewage influent was 2 to 94.8 ng/L and showed how part of the substance was adsorbed on the activated sludge through the treatment.

The point of this study is to focus, first, the

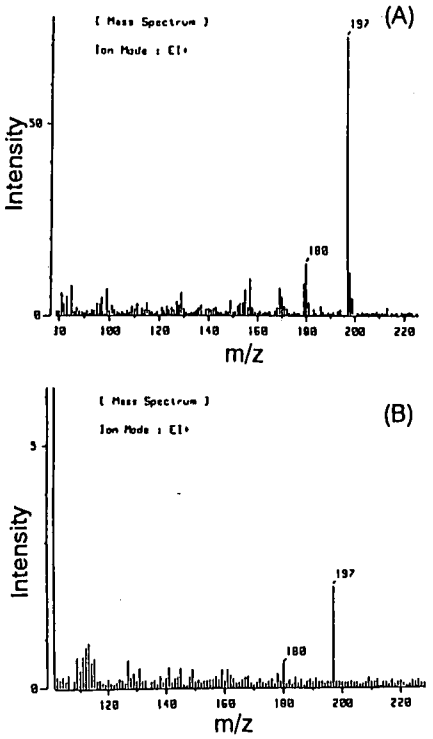

Fig. 5 Mass Spectra of authentic Trp-P-2 (A) and the purified Trp-P-2 (B) in the third-step HPLC fractions isolated from the blue-rayon extracts of the effluent from the municipal sewage treatment plant.

exstence of the heterocyclic amines dealing with human waste, second its procedure of eluate and detection by bioassay, and third the analysis of the compounds. So, we adopted 4 steps' procedures to identify the compound: (1)blue rayon extract for heterocyclic compounds, (2)*umu*-test NM2009 system for heterocyclic amines, (3)HPLC analysis by using ODS column for heterocyclic structures and (4)MS analysis. In the process of identification of the genotoxic substances in the effluent from a treatment plant dealing with the disposal of human waste, HPLC and MS analysis were earned out, respectively. The concentration of the Trp-P-2 identified in the effluent from this plant was taken as being 1 μ g/L[8].

In this research, we showed that Trp-P-2 and Trp-P-1 had been identified in the effluent taken from a municipal sewage treatment plant even after the physical and biological treatment process were conducted. The concentration of the Trp-P-2 was calculated as 10^{-2} μ g/L which is 1/100 of was obtained in the effluent from the secondary sedimentation tank found in the treatment plant concerned with the disposal of human waste. From the dose response of the sample, **Fig. 1** and the dose response of Trp-P-2 standard, 1 litre of the effluent has genotoxicity corresponded to about 5 to 6 μ g of Trp-P-2. The concentration of Trp-P-2 was actually 10^{-2} μ g/L, which is 1/500 of the "total genotoxicity" obtained the elute procedure. It seems that there are other many substances related to polycyclic aromatic amines having genotoxicity, because our procedure could obtain the parts of these genotoxic compounds. Our study concentrated on the possibility of the existence of carcinogenic substances in the effluent from the municipal sewage treatment plant where the treated water is drained back to the watershed which is usually the source of drinking water or used in a cultural area. With a lack of water source in urban area in Japan, the effluent of sewage treatment plants become to be used as toilet flushing, process water such as boiler and cooling, groundwater recharge and streamflow. In an attempt to understand the needs of advanced treatment before wastewater is reused, and to aim to regulate or to control the quality of the reused wastewater with its items of toxicity, investigation and piling up of the toxicity data should be carried into effect.

5. CONCLUSIONS

The substances inducing genotoxic activity in the effluent from a municipal sewage treatment plant are identified.

(1)High genotoxicity in the effluent could be observed. This activity is thought to be the inducement of aromatic amines.

(2)The fraction of genotoxicity related to aromatic amines are analyzed by HPLC. 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2) and 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1) are eluted by this fractionation.

(3)Trp-P-2 was identified by using MS analysis. The concentration in the effluent was calculated 10^{-2} μ g/L that was 1/100 in the effluent from a treatment plant of human waste. The trace of Trp p-2 was estimated to be originated to human waste partially.

Abbreviations and CAS Numbers:

2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ,CAS No.76180-96-6) , 2-amino-3,4-dimetylimidazo[4,5-*f*]quinoline (MeIQ,77094-11-2) , 2-amino-3,8-dimetylimidazo[4,5-*f*]quinoxaline (MeIQx,77500-04-0) , 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1,75104-43-7) ,3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2,72254-58-1) , 2-amino-3-methyl-9*H*-pyrido[2,3-*b*]indole (MeA α C,68006-83-7) , 2-amino-6-methyl-dipiyrido[1,2-*a*:3',2'-*d*]imidazole (Glu-P-1,67730-11-4)

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下水２次処理水中の癌原性 Trp-P-2, -1 の抽出

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水中の遺伝毒性を示す物質の同定に *umu*-testとＨＰＬＣ分析，ＭＳ分析を併用しその定量を試みた．ヘテロサイクリックアミンは，ヒトの発癌性物質と推定されている物質群で，ヒトの排泄物や雨水中に検出されている．それらの起源は，蛋白質を含む食品，あるいは植物（煙草の葉や木）を加熱処理したときに同定されている．我々は日本のある都市下水処理場の処理水中にヘテロサイクリックアミンの一種である 3-ｱﾐﾉ-1-ﾒﾁﾙ-5H-ﾋﾟﾘﾄﾞ[4,3-b]ｲﾝﾄﾞｰﾙ(Trp-P-2)を単離，MS 分析により同定し，その濃度が 10^{-2} μg/Lのｵｰﾀﾞｰであると推定した．