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Mutagenicity of the Sunlight-exposed Sample of Pyrene in *Salmonella typhimurium* TA98

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The organic matter derived from airborne particulate matter has been well known to be mutagenic in the Salmonella/microsome mutagenicity test. We and other investigators demonstrated that more than half of the mutagenicity of the acidic and the oxygenated fractions of the organic matter is due to directly active mutagens which require no metabolic activation (Pitts et al. 1977, Teranishi et al. 1978, Tokiwa et al. 1980). Wang et al. (1978) suggested that the source of these directly active mutagens may be automobile exhaust. However, some polyaromatic hydrocarbons (PAHs) are known to be transformed to directly active mutagen by irradiation with ^{60}Co gamma ray (Gibson et al. 1978) or by a reaction with gaseous pollutants such as nitrogen dioxide (Pitts et al. 1978, Tokiwa wt al. 1981). Also, McCoy et al. (1979) reported that exposure of environmental pollutants to visible light resulted in the formation of products endowed with direct-acting mutagenic and DNA-modifying activities. In addition to these chemical and biochemical transformations of PAHs in simulated conditions, it is reported that PAHs associated with airborne particles are readily photooxidized in real atomosphere (Pierce and Katz 1976) and the biological importance of their decomposition products was suggested (Tebbens et al. 1971).

These studies prompted us to investigate a relation between the photodegradation of PAHs in the real atomospheric environment and the resultant mutagenicity of their degradation products. In this paper, we describe the transformation of pyrene into directly active mutagen following exposure to sunlight in the real atomospher and the presence of directly active mutagens in the oxygenated and acidic fractions of the exposed sample of pyrene.

MATERIALS AND METHODS

Pyrene (99.5%, GC, Tokyo Kasei Kogyo Co., Ltd., Japan) was dissolved in benzene and deposited (ca. 58 mg and ca. 43 mg) on filter papers (23 x 17 cm, Filter paper No.50, Toyo Roshi, Tokyo, Japan). After the solvent had been evaporated, the filter paper was fixed on a wooden frame and exposed to sunlight in the ambient air on the roof of our institute for three days (10:00 - 16:00, August 23 - 25 and September 1 - 3, 1980). The PAH-laden filter was turned over every 1 h throughout the exposure period so that pyrene deposited on the

two sides of the filter paper could be equally deposed to the light.

The exposed sample was extracted with 250 ml of a mixture of benzene and methanol (4:1, v/v) by a Soxlet extractor for 8 h. The solution was filtered and evaporated to dryness at 40°C under reduced pressure. The residue was fractionated into the parent pyrene, the oxygenated and the acidic fractions as follows. The sample was transferred quantitatively to a separatory funnel using 50 ml of diethyl ether and a same volume of 1 N aqueous sodium hydroxide. It was shaken for 5 min. The aqueous layer was transferred to another funnel and re-extracted with 50 ml of diethyl ether. The ether layer was combined, washed with s small amount of water, dried over sodium sulfate and evaporated to dryness. The residue was adsorbed on a small amount of silicagel and transferred to the top of a column packed with 10 g of silicage (Wakogel C-200, activated at 110°C for 2 h). The column was first eluted with 60 ml of benzene, followed by an equal volume of a mixture of chloroform and methanol (1:2, v/v). The benzene eluate contained the parent pyrene fraction and the chloroform-methanol eluate the oxygenated fraction. The aqueous layer was acidified with 20 ml of 6 N hydrochloric acid and extracted twice with 50 ml of diethyl ether. The ether layer was washed with water, dried with sodium sulfate and evaporated to dryness. The residue contained the acidic fraction.

Pyrene was chemically oxidized by the method of Fatiadi (1965) and fractionated by the above described method.

The oxygenated and the acidic fractions of the sunlight-exposed sample of pyrene were further separated by high performance liquid chromatography (HPLC). HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph equipped with two Model 6000 solvent delivery systems, a Model 660 solvent programmar, a Model U6K injector and a Model 440 absorbance detector set at 254 nm using a 30 cm x 7.8 mm I.D. μBondapak C_{18} semipreparative column (Waters Assoc.). The column was eluted with a linear gradient of 60% to 80% methanol (HPLC grade, Wako Pure Chemicals, Osaka, Japan) in glass-distilled water in 15 min for the separation of the oxygenated fraction and eluted with 80 % methanol in water for the acidic fraction. A flow rate of 2.5 ml/min was used and the separation was carried out at room temperature.

The mutagenicity test with Salmonella typhimurium was performed as described by Ames et al. (1975). Tester strain TA98 was used because the PAH derivatives formed by various radiations in air had been found to be frameshift mutagens (Gibson et al. 1978). The tester strain was isolated from a histidine enriched minimal glucose agar plate containing ampicillin in a concentration of 25 μg/ml, and grown in nutrient broth (Difco) overnight at 37°C with shaking. The sunlight-exposed sample and its three fractions were dissolved in a suitable amount of dimethyl sulfoxide (DMSO). An aliquot (0.1 ml) of the DMSO solution was added to a test tube

containing 2 ml of the top agar and 0.1 ml of a fresh overnight culture of the tester strain, then 0.5 ml of S9 mix or 0.1 M phosphate buffer (pH 7.4) was added. The contents were mixed and poured onto a minimal agar plate.

A 9000 x g supernatant (S9) was prepared from the liver of male rats treated with both phenobarbital and dibenzo(ah)anthracene (Teranishi et al. 1978).

RESULTS AND DISCUSSION

In order to investigate the relation between the degradation of PAHs in the real atomospheric environment including solar radiation and the mutagenicity of their degradation products, pyrene was selected for experimentation; because (1) it is not mutagenic both with and without enzymatic activation, (2) it always appears in airborne particulate matter, (3) the mutagenic activity of its gamma ray-irradiated sample and its reaction products with nitrogen dioxide is reported to be very high (Gibson et al. 1978, Tokiwa et al. 1981).

Pyrene was exposed to sunlight in the real atomosphere for 18 h in three days during the summer months in 1980. The exposed sample was extracted and separated into three fractions by acid-base partitioning and by silicagel column chromatography. It was found that average yield of the conversion of pyrene to the oxygenated compounds was 16% and that to the acidic was 9% (Table 1). Pitts et al. (1978) reported that benzo(a)pyrene (B(a)P) was transformed

Table 1. Recovery in each fraction of the exposed sample of pyrene

Fraction	Experiment-1 weight		Experiment-2 weight		Average
	mg	%	mg	%	%
Crude mixture	55.2	100	40.2	100	100
Parent pyrene	39.2	71.0	28.5	70.9	71.0
Oxygenated	6.9	12.5	7.9	19.6	16.1
Acidic	5.0	9.1	3.7	9.1	9.1
Total	51.1	92.6	40.1	99.6	96.2

Pyrene (ca. 58 mg or ca. 43 mg) was deposited on a filter paper (23 x 17 cm) and exposed to sunlight in the ambient air for 3 days (10:00 - 16:00, August 23 - 25 and September 1 - 3, 1980).

by the exposure to ambient smog in the dark into B(a)P-quinones (major products) and hydroxy, dihydroxy and dihydrodiol derivatives (minor products). Tebbens et al. (1977) proposed that the photooxidation of B(a)P first yields polycyclic quinones and further photooxidized to the corresponding carboxylic acids. As the fractionation step in our study, the alkaline solution of the acidic fraction showed a typical dark brown color, that disappeared on acidification. This behaviour can be expected for compounds of polyphenolic nature. These findings suggested that the oxygenated fraction may contain pyrene-quinone(s) and the acidic fraction may contain polyphenolic compound(s) and/or carboxylic acid(s).

The exposed sample and its three fractions were tested for mutagenicity and the results are shown in Table 2. In three fractions tested the highest activity was found in the acidic fraction, and it possessed about 7 times higher activity per unit weight than that of the oxygenated fraction both with and without S9 mix. In addition, almost all the mutagenic substances in the two active fractions seemed to be directly active type, because the activity of both fractions was not enhanced by the addition of S9 mix. With respect to the contribution of the mutagenicity of each fraction to the whole mutagenicity of the photooxidized sample of pyrene, the acidic fraction plays important role in the mutagenicity of the sample.

Table 2. Mutagenic activity of the each fraction of the exposed sample of pyrene

Fraction	Amount (μg/plate)	Revertant colonies per plate(*) With S9 mix	Without S9 mix
Control (DMSO)		17	11
Crude mixture	1500	1708	2664
Parent pyrene	2000	30	56
Oxygenated	500	181	287
Acidic	500	1362	1820

(*), TA98 was used. Each value is the average of duplicate plates.

The oxygenated and the acidic fractions were further separated by HPLC using a reversed phase column. Fig. 1 shows the typical chromatograms of the oxygenated (A) and the acidic (B) fractions. The oxygenated fraction, 200 μg, was injected onto the HPLC column and separated by a gradient elution method and 500 μg of the acidic fraction was separated isocratically at 80% methanol in water. The eluate from the peaks was collected and the solvent was evaporated. The residue was dissolved in DMSO and the mutagenicity was tested. The results are shown in Fig. 2A and 2B. The oxygenated fraction was separated into 9 primary peaks (Fig. 1A) and the material from

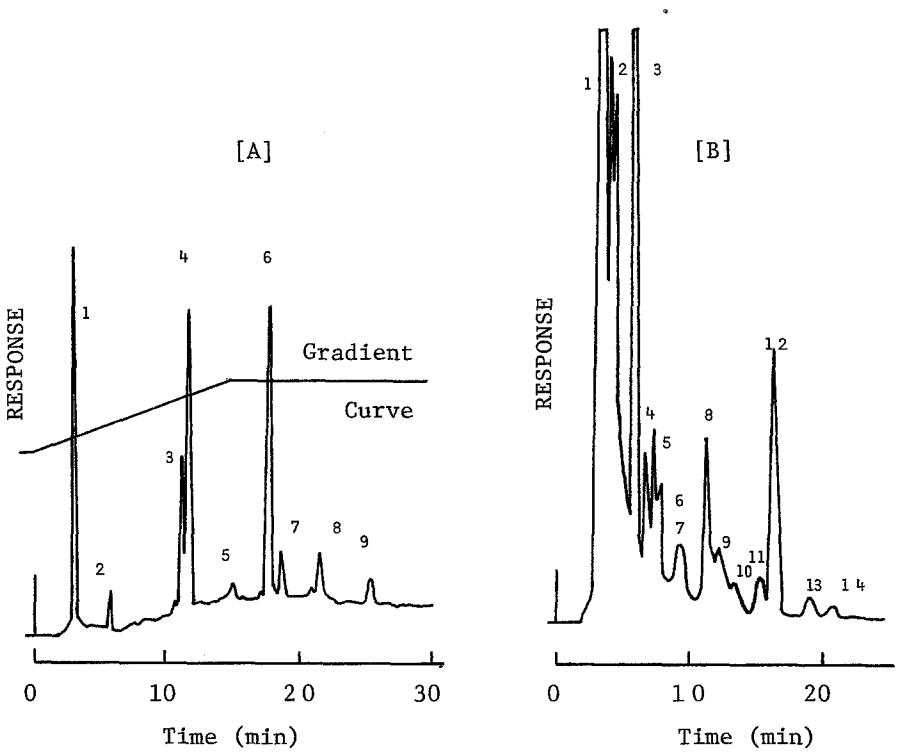

Fig. 1. High performance liquid chromatograms of the oxygenated [A] and the acidic fractions [B] of the exposed sample of pyrene. Amount injected: [A] 200 µg, [B] 500 µg; HPLC conditions: column, µBondapak C_{18} (7.8 mm x 30 cm); flow rate, 2.5 ml/min; detection wave length, 254 nm; mobile phase, [A] 60% to 80% methanol in 15 min, [B] 80% methanol in water; A.U.F.S., [A] 0.2, [B] 2.0; temperature, ambient.

peak No.6 was found to be mutagenic without S9 mix (Fig. 2A). Its activity in the presence of S9 mix was reduced to one fourth in the absence of S9 mix. The result indicates that the directly active mutagen(s) in peak No.6 was partially inactivated by S9. The acidic fraction contained at least 14 compounds (Fig. 1B) and the materials from 11 peaks among them were mutagenic without S9 mix (Fig. 2B). The directly active mutagens in the acidic fraction were also found to be inactivated by the addition of S9 mix. Under these chromatographic conditions, as materials from one peak overlapped into adjacent peaks, it is not possible to determine with certainty the number of mutagenic compounds in the acidic fraction.

As the chmical nature of the mutagenic species formed in these experiment has not been fully discovered, possible reference compounds on the photochemical oxidation of pyrene were prepared chemically by the method of Fatiadi (1965). The oxidation mixture, in which main products have been known to be 1,6- and 1,8-pyrenediones, was

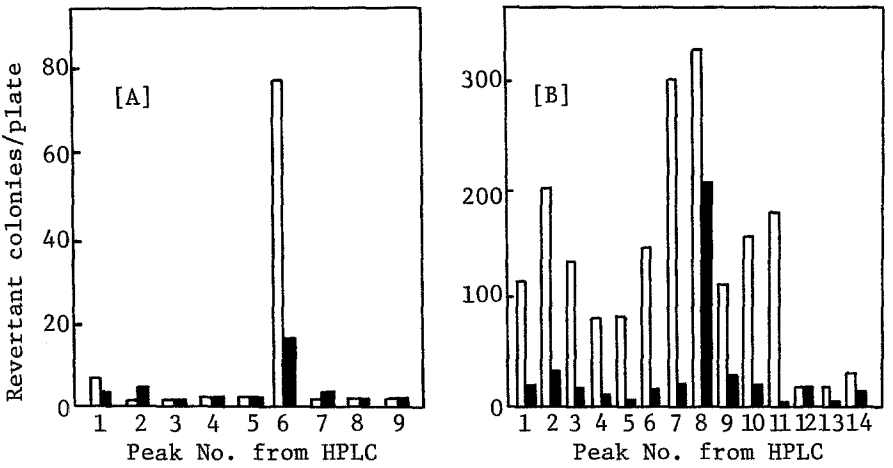

Fig. 2. Mutagenic activity of the oxygenated [A] and the acidic [B] fractions of the exposed sample of pyrene. The eluate from the peaks in Fig. 1. was collected, the solvent evaporated and the residue re-dissolved in DMSO. Mutagenicity was tested in TA98 both with and without S9 mix in duplicate. Spontaneous revertants, 18 with S9 mix (▬) and 19 without S9 mix (▭), were subtracted.

Table 3. Mutagenic activity of the chemically oxidized sample of pyrene by $K_2Cr_2O_7$ and H_2SO_4

Fraction	Amount (μg/plate)	Revertant colonies per plate(*) With S9 mix	Without S9 mix
Control (DMSO)		18	10
Oxidized sample	500	20	5
Oxygenated	500	20	8
Acidic	500	20	11

(*), TA98 was used. Each value is the average of duplicate plates.

fractionated by the above described method and tested for mutagenicity. The crude mixture, the acidic and the oxygenated fractions that contains the above two pyrenediones, were found not to be mutagenic both with and without S9 mix (Table 3). This result suggests that the directly active mutagen(s) in the oxygenated fraction of the photooxidized sample of pyrene are not these two pyrenediones. According to Gibson et al. (1978) and Pitts et al. (1978), four dione derivatives of B(a)P (1,3-; 1,6-; 3,6- and 6,12-) were also not mutagenic in the Salmonella/microsome mutagenicity test.

It is known that PAH react with nitrogen dioxide in the dark and form direct-acting mutagenic nitroderivatives (Pitts et al. 1978, Tokiwa et al. 1981). The present study indicates that the acidic and the oxygenated compounds possessing directly active mutagenic activity can be formed from non-mutagenic parent substance in the actual atomospheric environment including solar-radiation. However, the mode of formation of directly active mutagens in photochemical oxidative products is not well known. Gibson et al. (1978) proposed that the radiation-induced mutagenic activity, though independent upon the frequency of the exciting radiation, for visible and UV light as well as the more energeric ^{60}Co gamma ray, was dependent upon oxygen. On the contrary, McCoy et al. (1979) reported that photo-induced direct-acting mutagenic activity increased when a singlet oxygen generation was blocked, when PAH had been illuminated in DMSO solution with fluorescent white light.

Thus, the mutagenic substances such as the acidic and the oxygenated compounds described here and nitroderivatives (Pitts et al. 1978, Tokiwa et al. 1981) have been found to be formed by the exposure of PAHs to sunlight and/or to gaseous pollutants in the ambient atomosphere. Therefore, we emphasize that the toxicological studies should be conducted not only on the parent cahmicals but also on their environmental degradation products formed by various kinds of gaseous pollutants, sunlight, UV light and so on, as well as those by enzymatic activation.

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