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Mutagen Formation and Nitration by Exposure of Phenylphenols to Sunlight in Water Containing Nitrate or Nitrite Ion

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Mutagens have been ascertained to be present in river sediments (Suzuki et al., 1982a; Sato et al., 1983, 1985). Their mutagens in aquatic environments are considered to be attributable not only to the inflow of industrial effluent and domestic sewage containing mutagens but also chemical and biological transformations of organic pollutants to mutagens. In earlier studies, we found that UV irradiation causes mutagen formation in aqueous solution of aromatic compounds in the presence of nitrate or nitrite ion (Suzuki et al., 1982b, 1983, 1985, 1988). The mutagen was proven to be formed through nitration, nitrosation, hydroxylation and oxidation to a quinone of the parent aromatics with radicals of NO, NO_2 and OH derived from photolysis of nitrate or nitrite ion. Aquatic environments are well known to be polluted by various aromatic compounds (Pitt et al., 1975; Basu et al., 1978). Nitrite ion also exists in river, lake and sea waters, though the concentration is very low level (Brinkhoff, 1978; Okada et al., 1979; Zafiriou et al., 1980). The effluents from night-soil treatment plant and sewage treatment plant are known to contain very high concentration of nitrite (Kaiga et al., 1984).

This information suggests the high possibility that a photochemical reaction of aromatics with nitrate or nitrite ion may be a important cause of the mutagens in aquatic environment. However, it is still uncertain whether a photochemical mutagen formation is caused by sunlight because the earlier studies were mostly undertaken by irradiating UV light derived from a high-pressure mercury lamp. To clarify this point we designed a experiment regarding exposure to sunlight.

In this paper, we substantiate that the mutagen formation and nitration actually occur when phenylphenols,

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which are used for storage of citrus fruits as a fungicide, are exposed to sunlight.

MATERIALS AND METHODS

Phenylphenols were obtained from the Tokyo Kasei Co., sodium nitrite, sodium nitrate and diethyl ether were from the Kanto Kagaku Co. All these chemicals were of the best grade commercially available and were used without further purification. 2-Hydroxy-3-nitrobiphenyl (2H3NBP) and 4-hydroxy-3-nitrobiphenyl (4H3NBP) were synthesized and purified according to the method described in the previous paper (Suzuki et al., 1985).

The silica gel carrier (Wako gel Q22, 200 mg) coated with o- or p-phenylphenol (Ca. 10 mg) was suspended in 200 ml of neutral aqueous nitrite (NO_2-N, 16.2 ppm) or nitrate (NO_3-N, 16.5 ppm) solution in a Erlenmeyer flask with stopper (made of Pyrex glass, 200 ml). The flasks were placed out door in summer and exposed to sunlight over periods of 2 to 8 weeks. One of the same flask was placed in a dark room for 8 weeks as control.

Phenylphenols and their nitrated products, 2H3NBP and 4H3NBP, in the exposed solution were extracted with ether and then analyzed by means of high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: column (at 50 °C), Zorobax ODS (4.6 mm i.d. x 150 mm); solvent, CH_3OH-H_2O gradient (CH_3OH 45-75%/20 min); flow rate, 1 ml/min; wavelength for detection, 254 nm; internal standard, α-naphthoquinone.

The bacterial strain used for the mutation assay was Salmonella typhimurium TA98. The mutation test was performed by Ames' method (Maron and Ames, 1983) with some modifications including a step of preincubation of test materials with tester strain for 20 min at 37 °C. Liver S9 fractions used were prepared from the liver of PCB-treated male rats as described by Maron and Ames (1983). Each sample (the ether extract of the exposed solution) was dissolved in methanol (1 mg/ml) after evaporation and a 0.1 ml each of the solution (per one plate) was subjected to the assay. Each sample was assayed with 4 replicate plates.

RESULTS AND DISCUSSION

Phenylphenols were exposed to the sunlight for 2 to 8 weeks in summer in neutral aqueous nitrate and nitrite solutions. The yield of the ether extract obtained from the exposed solution, the amounts of phenylphenol degraded and of nitrophenylphenol produced, and the mutagenicities of the ether extract toward S. typhimurium TA98 in the absence and presence of rat liver microsomal fraction (S9) were summarized in Tables 1 to 4.

As shown in Table 1, o-phenylphenol in aqueous nitrate solution was completely degraded by exposure for 14 days, although when placed in a dark room, there was little degradation even after 56 days. However, neither nitrophenylphenol i.e., 2H3NBP, nor mutagenicity of the ether extract was detected, indicating that neither nitration nor mutagen formation occurred.

On the other hand, in aqueous nitrite solution shown in Table 2, production of 2H3NBP and mutagen formation were observed. Both the largest amount of 2H3NBP and the highest mutagenicity of the ether extract were

Table 1. Mutagen formation and nitration by exposure of o-phenylphenol to the sunlight in aqueous nitrate solution[a)]

Exposure time	Ether extract	Photolysis		Mutagenicity of extract[b)] (Revertants/100 μg/plate)	
		o-PP degraded	2H3NBP produced		
(days)	(mg)	(nmoles)	(nmoles)	S9 (-)	S9 (+)
14	5.7	54.6	ND[c)]	28	47
28	3.6	54.6	ND	23	58
56	3.4	54.6	ND	39	50
dark[d)]	12.1	10.7	ND	64	65

a) The solution (200 ml) contained 9.3 mg (54.6 nmoles) of o-phenylphenol (o-PP) and 20 mg of sodium nitrate.
b) Spontaneous revertants of S. typhimurium TA98 were 44 and 48 in without S9 and with S9, respectively.
c) ND means not-detectable.
d) The solution was placed in a dark room for 56 days.

Table 2. Mutagen formation and nitration by exposure of o-phenylphenol to the sunlight in aqueous nitrite solution[a)]

Exposure time	Ether extract	Photolysis		Mutagenicity of extract[b)] (Revertants/100 μg/plate)	
		o-PP degraded	2H3NBP produced		
(days)	(mg)	(nmoles)	(nmoles)	S9 (-)	S9 (+)
14	5.7	53.3	1.6	362	161
28	4.7	54.6	0.4	240	127
56	2.6	54.6	0.2	184	120
dark[c)]	11.2	17.5	0.3	32	67

a) The solution (200 ml) contained 9.3 mg (54.6 nmoles) of o-phenylphenol (o-PP) and 16 mg of sodium nitrite.
b) Spontaneous revertants of S. typhimurium TA98 were 44 and 48 in without S9 and with S9, respectively.
c) The solution was placed in a dark room for 56 days.

observed in the samples exposed for 14 days, although only a few percentage of o-phenylphenol was remained in the solution. And both of them decreased by further exposure. These results indicate that either nitration or mutagen formation occurred during early periods of the exposure. The amount of 2H3NBP and the mutagenicity of the ether extract probably reached maxima by shorter exposure than 14 days. In the solution placed in a dark room, a slight amount of 2H3NBP was detected, although the ether extract exhibited no mutagenicity and most of the o-phenylphenol was remained. This may

Table 3. Mutagen formation and nitration by exposure of p-phenylphenol to the sunlight in aqueous nitrate solution[a)]

Exposure time	Ether extract	Photolysis		Mutagenicity of extract[b)] (Revertants/100 μg/plate)	
		p-PP degraded	4H3NBP produced		
(days)	(mg)	(nmoles)	(nmoles)	S9 (-)	S9 (+)
14	4.0	46.7	0.0	43	55
28	3.8	46.7	0.1	45	53
56	3.1	46.7	ND[c)]	33	48
dark[d)]	11.7	0.4	ND	31	77

a) The solution (200 ml) contained 7.9 mg (46.7 nmoles) of p-phenylphenol (p-PP) and 20 mg of sodium nitrate.
b) Spontaneous revertants were 44 and 48 in without S9 and with S9, respectively.
c) ND means not-detectable.
d) The solution was placed in a dark room for 56 days.

Table 4. Mutagen formation and nitration by exposure of p-phenylphenol to the sunlight in aqueous nitrite solution[a)]

Exposure time	Ether extract	Photolysis		Mutagenicity of extract[b)] (Revertants/100 μg/plate)	
		p-PP degraded	4H3NBP produced		
(days)	(mg)	(nmoles)	(nmoles)	S9 (-)	S9 (+)
14	6.0	41.7	1.2	128	97
28	4.2	41.7	0.2	91	77
56	3.8	46.7	0.1	53	50
dark[d)]	11.4	7.3	ND[c)]	25	74

a) The solution (200 ml) contained 7.9 mg (46.7 nmoles) of p-phenylphenol (p-PP) and 16 mg of sodium nitrite.
b) Spontaneous revertants of S. typhimurium TA98 were 44 and 48 in without S9 and with S9, respectively.
c) ND means not-detectable.
d) The solution was placed in a dark room for 56 days.

be due to exposure to a slight light leak during the long exposure period. This fact seems to indicate that the nitration of o-phenylphenol occurs very easily in aqueous nitrite solution.

Tables 3 and 4 show the results of nitration and mutagen formation in p-phenylphenol in aqueous nitrate and nitrite solutions, respectively. In a similar manner as o-phenylphenol, p-phenylphenol also produced mutagens only by exposure in the aqueous nitrite solution. A slight amount of nitrophenylphenol (4H3NBP) was detected even in the aqueous nitrate solution, although the amount was much smaller than that in the nitrite solution. This indicates that photochemical nitration by the sunlight is possible to occur even in nitrate solution. The solution placed in a dark room showed that neither nitration nor mutagen formation occurred in both nitrate and nitrite solutions, though a small amount of p-phenylphenol was degraded.

As mentioned above, phenylphenols were substantiated to be nitrated and to be converted to a mutagen by exposure to sunlight in water containing nitrite ion. The mutagen formed was direct-acting as shown by the fact that the mutagenicities of the ether extracts were depressed with metabolic enzymes in rat liver S9. The mutagenicities of the ether extracts were proportional to the amounts of nitrophenylphenols produced, suggesting that the mutagen formation depended on nitration of phenylphenols. Since neither 2H3NBP nor 4H3NBP is mutagenic toward S. typhimurium TA98, the mutagen seems to be a polynitro-phenylphenol. This is also supported by the facts that many nitro-aromatic compounds are direct-acting mutagens (Rosenkranz and Mermelstein, 1983) and that the mutagens produced from biphenyl by UV irradiation in aqueous nitrate solution were identified to be di-nitrophenylphenols (Suzuki et al., 1985).

On the other hand, it was also substantiated that photochemical mutagen formation by exposure to sunlight did not occur in aqueous nitrate solution and that the nitration of phenylphenols in aqueous nitrate solution was slow, compared with that in aqueous nitrite solution. This can be explained by the following mechanisms which were proposed in our earlier studies on photochemical reaction (Suzuki et al., 1988). The nitration of phenylphenols and hence the mutagen formation are dependent on radicals, NO and NO_2 originated from photolysis of nitrate and nitrite ions. Nitrate and nitrite ions in aqueous solution show UV absorptions with maxima at 300 nm and 355 nm, respectively. And the UV absorptions cause excitation and subsequent photolysis of nitrate and nitrite ions to give NO and NO_2 radicals. In addition, the sunlight

includes long wavelength light but not sufficient short wavelength light to excite nitrate ion.

The degradations of phenylphenols were observed in the same degree in either nitrate or nitrite solution, indicating that the photolysis of phenylphenols themselves other than nitration was independent of the photolysis of nitrate and nitrite ions. This implies that the nitration and the mutagen formation are governed only by photolysis of nitrate and nitrite ions and independent of the photo-degradation of phenylphenols. Consequently, it is concluded that the most important factor for photochemical mutagen formation in aquatic environment is the concentration level of nitrite ion.

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