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A Screening Method for Detection of Aromatic Amines in Environmental Samples Based on Their Methemoglobin Formation Activities *in vitro*

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Many kinds of organic pollutants are known to be present in aquatic environment. The occurrence of skin melanoma and fin erosion of certain fish have been considered to be attributable to these pollutants (KIMURA 1976; KINAE et al. 1981; WELLINGS et al. 1976). It is very important from ecotoxicological point of view to clarify what class of pollutants causes such injuries in water organisms. The screening method for detection of certain classes of pollutants based on their specific biological activity (DREVENKAR et al. 1981; BRADLAW & CASTERLINE 1979; Suzuki et al. 1982) seems to be a useful tool to clarify the cause of injury.

Aromatic amines, many of which are metabolized in vivo to cause methemoglobinemia and/or carcinoma (KIESE 1966; WEISBURGER & WEISBURGER 1973), are produced in large quantities as intermediates in the manufacture of a wide variety of dyes. Some of these aromatic amines have been detected in aquatic environment (NELSON & HITES 1980; WEGMAN & KORTE 1981). In addition, certain aromatic nitro pesticides are known to be easily converted to the corresponding amines in environment (NAKAGAWA & CROSBY 1975). No attempt, however, has been made to develop a biological or biochemical screening method for detection of aromatic amines so far as we are aware.

In this paper, we examined the possibility of a screening method for detection of aromatic amines on the basis of methemoglobin-formation reaction in vitro

MATERIALS AND METHODS

Aromatic amines, aliphatic amines, aromatic nitro compounds, di(2-ethylhexyl)phthalate, phenol and octachlorobiphenyl were purchased from Tokyo Kasei Co. (Tokyo), Nakarai Chemicals Co. (Kyoto), Kanto Kagaku Co. (Tokyo) and Wako Pure Chemical Co. (Osaka). Malathion and BHC (1,2,3,4,5,6-hexachlorocyclohexane gamma isomer: 40%, other isomers: 60%) were purchased from Polyscience Co. (Illinois). These chemicals were of the best grade commercially available, and were used without further purification.

Defibrinated sheep blood was obtained from Nippon Seibutsu Zairyo Center (Tokyo). The blood (50 mL) was centrifuged for 20 min to separate cells and plasma. The erythrocytes were washed successively with isotonic buffer (KOIZUMI et al. 1964), and finally suspended in 300 mL of the same buffer.

To a L-shape test tube containing 5 mL of erythrocytes suspension, 0.1 mL of 9000Xg supernatant of rat liver homogenate (S9), 0.1 mL of nicotinamide (120 μmole), 0.1 mL of glucose-6-phosphate (60 μmole), 0.1 mL of NADP (1.8 μmole), 0.1 mL of $MgCl_2$ (60 μmole) and 0.2 mL of the isotonic buffer was added 0.1 mL of dimethyl sulfoxide (DMSO) solution of a test compound. The above mixture was incubated in a shaking water bath at 37°C. After 1 h, 1-mL aliquot of the resulting mixture was mixed with 4 mL of 0.05 M phosphate buffer (pH 7.3) and 1 mL of 0.5% saponin. Cell membranes were removed by centrifugation (10000Xg, 10 min), and change in optical density at 630 nm of the supernatant solution was measured (EVELYN & MALLOY 1938). Methemoglobin-formation activity was expressed as methemoglobin (%). A control was run similarly in the absence of a test compound, and its methemoglobin (%) was subtracted from a test compound.

The S9 was prepared from PCB-treated male rats (Sprague-Dawley) as described previously (Ames et al. 1975).

Sandy or muddy sediments were collected at 4 points located from upper reaches to lower reaches of Ayase river [Shimo-Hasuda (Hasuda city), Sato (Kawaguchi city), Yanaginomiya (Yashio city) and Horikiri (Katsushika-ku, Tokyo)], and centrifuged for 15 min at 3000Xg to remove excess water. The sediment samples were then mixed well, and 20~30 g portion of each sample was extracted with 100 mL of 2-propanol for 24 h using a Soxhlet apparatus. Another portion was dried at 110°C to measure its water content. The extracts were evaporated to dryness under reduced pressure at 40°C. The resulting residue was dissolved in DMSO to give the concentration of 25 mg/mL, and a 0.1-mL aliquot was subjected to the methemoglobin formation test. Amounts of 2-propanol extracts obtained by the above method were 113.4 (Shimo-Hasuda), 508.9 (Sato), 307.0 (Yanaginomiya) and 1114.5 mg (Horikiri) per 100 g of dry sediment.

RESULTS AND DISCUSSION

Methemoglobin-formation activities of certain aromatic amines have been tested *in vitro* using animal liver homogenates (CHOW & MURPHY 1975; MIYAUCHI et al. 1981). In the present study, we used the 9000Xg supernatant of liver homogenate (S9) of PCB-treated rats because PCB

induces microsomal oxidase enzyme systems which convert aromatic amines to N-hydroxylated derivatives that are responsible for methemoglobin formation (KIESE 1966; WEISBURGER & WEISBURGER 1973).

TABLE 1 shows the methemoglobin-formation activities of various compounds. All of the tested aromatic amines induced methemoglobin formation. Among these compounds, 2-aminofluorene showed the highest activity, while aniline showed the lowest activity. 1-Naphthylamine, 2-naphthylamine, 1-aminopyrene and benzidine showed the activity at a similar level. o-Aminodiphenyl showed a higher activity than the above group did, and 2-aminoanthracene showed a lower activity. No structure-activity relationships were observed among these compounds.

The methemoglobin-formation activity of ortho- or para-hydroxylated aniline was higher than that of aniline, while the reverse was the case with meta-hydroxylated aniline. An increase in activity by the presence of hydroxy substituent at ortho position was also observed in the case of 1-hydroxy-2-naphthylamine. Similar phenomenon in vivo has been reported with para-chloroaniline (NOMURA 1975), and in vitro with phenoxy substituted derivatives of aniline (MIYAUCHI 1981).

On the other hand, aromatic nitro compounds hardly induced methemoglobin formation except 1-nitronaphthalene which induced only about 10% methemoglobin formation even at high doses. Aromatic nitro compounds are reduced in vivo by nitroreductase to cause methemoglobinemia (MILLER et al. 1955; REDDY et al. 1976). The nitroreductase activity, however, is known to be inhibited completely by oxygen (STERNSON 1975). Therefore, low methemoglobin-formation activities observed with the tested nitro compounds are probably attributable to the aerobic experimental conditions employed.

Aliphatic amines and several typical environmental pollutants showed no activity as shown in TABLE 1.

The above-mentioned results indicate that in vitro methemoglobin-formation test is specific for aromatic amines. We attempted to use this test as a screening tool for detection of aromatic amines in river sediments.

2-Propanol extracts of the sediments of Ayase river, a typical urban river of Japan, were tested for their methemoglobin-formation activities. The activities of these extracts are shown in TABLE 2 together with the aromatic amine content estimated on the basis of the conventional coupling reaction with N-(1-naphthyl)ethylenediamine (NORWITZ 1982). The methemoglobin-formation activities of these samples ranged from about 10 to 20%

Table 1. Methemoglobin-formation activities of various compounds

Compound	Dose	Methemoglobin (%)
aniline	0.1	2.4
	1.0	14.1
o-hydroxyaniline	0.1	36.0
	1.0	55.2
m-hydroxyaniline	0.1	0.2
	1.0	1.8
p-hydroxyaniline	0.1	4.0
	1.0	30.6
1-naphthylamine	0.005	9.4
	0.01	15.2
	0.05	29.4
	0.1	34.3
2-naphthylamine	0.005	11.3
	0.01	16.9
	0.05	30.6
	0.1	34.4
1-hydroxy-2-naphthylamine	0.1	44.1
	0.5	69.1
2-aminoanthracene	0.005	7.1
	0.01	10.8
	0.05	13.8
	0.1	15.1
1-aminopyrene	0.005	13.6
	0.01	17.7
	0.05	22.2
	0.1	26.0
2-aminofluorene	0.005	27.9
	0.01	42.1
	0.05	58.2
	0.1	57.4
o-aminodiphenyl	0.005	7.8
	0.01	16.3
	0.05	47.3
	0.5	57.2
benzidine	0.005	3.8
	0.01	6.5
	0.05	25.1
	0.1	33.1
nitrobenzene	0.1	0.6
	1.0	1.5

1-nitronaphthalene	0.1	9.0
	1.0	11.4
	10.0	10.5
1-nitropyrene	0.1	8.9
	0.5	7.7
	1.0	9.2
2-nitrofluorene	0.1	1.4
	0.5	2.4
	1.0	1.7
o-nitrodiphenyl	0.1	0.7
	0.5	0.5
	1.0	0.5
n-butylamine	1.0	0
	10.0	0
n-hexylamine	1.0	0.2
	10.0	0.9
cyclohexylamine	5.0	0
	10.0	0
octachlorodiphenyl	1.0	0
BHC	1.0	0
malathion	1.0	0
di(2-ethylhexyl)-phtalate	1.0	1.0
phenol	1.0	0.6
	10.0	0.6

Table 2. Methemoglobin-formation activities of river sediment extracts and their aromatic amine contents estimated by the colorimetric method

Sampling point	[A] Methemoglobin (%)	[B] Aromatic amine content (μg)*	[A]/[B]
upper reaches			
↑			
Shimo-Hasuda	12.6	5.2	2.4
Sato	24.1	9.9	2.4
Yanaginomiya	18.4	3.0	6.1
Horikiri	17.0	2.0	8.5
↓			
lower reaches			

* 1-naphthylamine equivalents.

at the tested dose level. The decreasing order of activity of the extracts was Sato > Yanaginomiya > Horikiri > Shimo-Hasuda, while that of the aromatic amine contents estimated colorimetrically was Sato > Shimo-Hasuda > Yanaginomiya > Horikiri. There seems to be a rather good agreement between these orders considering that the difference in sensitivity of the methemoglobin-formation test to each of aromatic amines differs from that of the colorimetric method, suggesting that the methemoglobin-formation test specifically detects aromatic amines in sediment extracts.

Methemoglobin (%) per μg 1-naphthylamine equivalents is also shown in TABLE 2. The values of two points located at lower reaches were larger than those located at upper reaches, indicating that the aromatic amines showing higher methemoglobin-formation activities were contained in lower-reach sediments. This tendency is probably attributable to a qualitative change in contamination at lower reaches of the river.

The above results indicate that *in vitro* methemoglobin-formation test is applicable to various environmental samples, and that the method is probably useful as a screening tool for aromatic amines. Simultaneous use of a chemical screening method as attempted in the present study would enable us to obtain secure and valuable information.

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