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In vitro Cholinesterase Inhibition of Organic Matter in Urban and Rural River Sediment

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To measure the presence of toxic substances in aquatic environments, various toxicity tests have been carried out. For research done on environmental toxicities writers refer to several enzymatic studies. Fritschi et al.(1975) measured the inhibitory effect of river water on cholinesterase activity during water purification. Tyler (1974, 1976a, 1976b) investigated the influence of heavy-metal pollution on the activities of several hydrolytic enzymes in forest soil. The use of in vitro cholinesterase inhibition to detect organophosphate pesticides in surface water (Greve 1975) or the G-6-PDH inhibition to screen the contamination of petroleum effluents (Rutherford et al. 1979; Armant et al. 1980) has been proposed.

Latent toxicities cannot be found by chemical analyses of environmental samples, but the above mentioned bioassays make it possible to evaluate the direct latent toxicity of samples containing diverse compounds. This paper deals with the inhibitory effect of solvent extracts of urban and rural river sediment on cholinesterase in vitro.

MATERIALS AND METHODS

Sediment samples were collected from the surface layer in five urban rivers (21 May 1981), and five rural rivers (8 July 1981) by Ekman-Berge dredge (Figure 1). The Tsurumi, Meguro, Sumida, Ara and Ayase rivers, which are located near Tokyo, were chosen as the urban rivers. The rivers which run through the paddyfield region near Kujukuri beach were chosen as rural rivers.

The sediments were dried at room temperature and powdered. The samples obtained (dry weight 200 g) were extracted twice every 8 hr with a mixture of 200 ml n-hexane (Hex) and 100 ml distilled water by shaking

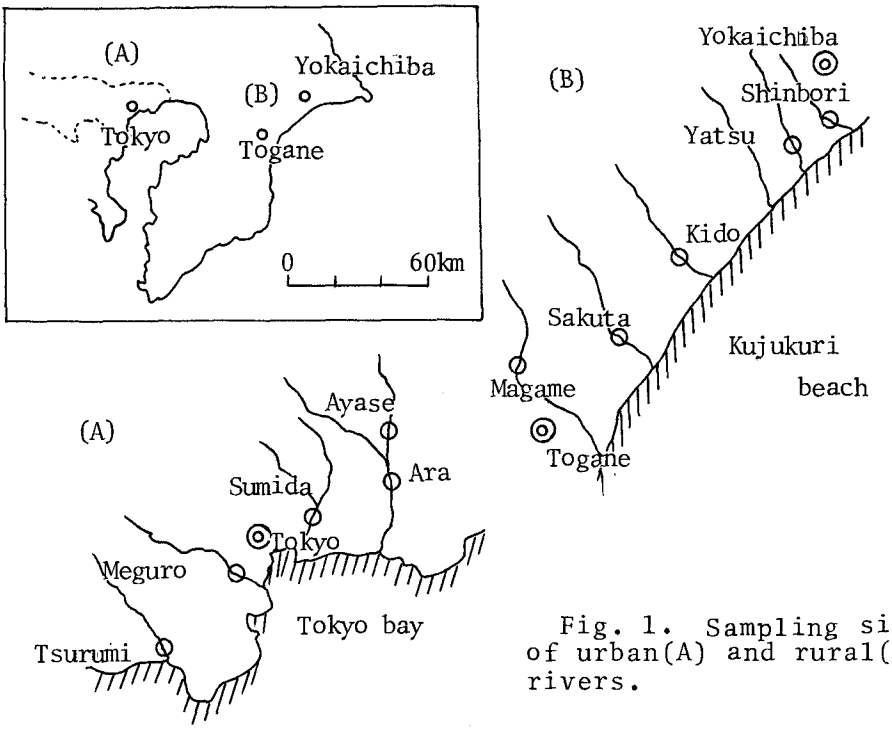

Fig. 1. Sampling sites of urban(A) and rural(B) rivers.

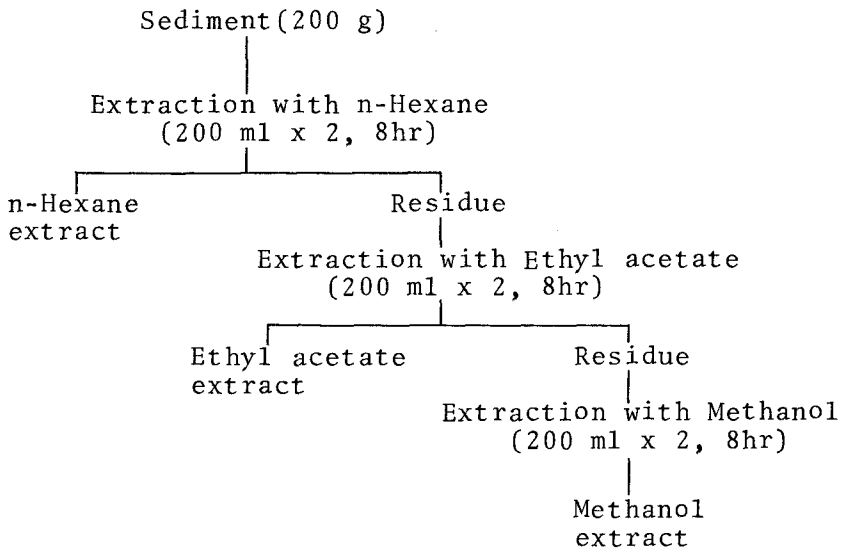

Fig. 2. Fractionating method of assay.

at room temperature. Next they were extracted using 200 ml ethyl acetate(EtAc) and 100 ml distilled water, and then 200 ml methanol(MeOH). Each solution was evaporated under reduced pressure below 40°C using a K.D. concentrator.

For cholinesterase assay, the fractionated samples were dissolved in dimethyl sulfoxide (DMSO) /ethyl acetate (4/1,v/v) for Hex and EtAc extracts, and DMSO for MeOH extracts. A portion 0.2 ml of sample solution and 0.8 ml enzyme solution (cholinesterase butyryl, Sigma, 1 mg /15 ml 0.067 M phosphate buffer, pH 8) were mixed, and the mixture was preincubated for 30 min at 30°C. A 0.1 ml aliquot of the preincubated solution was added to the mixture of 2.7 ml of 0.067 M phosphate buffer and 0.1 ml 6 x 10^{-3} M 5,5-dithio-bis-(2-nitrobenzoic acid) solution, and incubated for 5 min at 30°C. Then 0.1 ml of 0.15 M acetyl thiocholine iodide was added, and adsorption was measured at 416 nm by spectrophotometer. The inhibition rate caused by extract was calculated by comparing the enzyme activity without sediment extract.

Thin-layer chromatography for each extract was performed using the plates which were coated with a 0.25 mm layer of Kieselgel GF 254 Type 60 (E.Merk) activated at 110°C for 1 hr. The plates were developed with n-hexane/ethyl acetate (1/1,v/v) for Hex extract, ethyl acetate/methanol (1/1,v/v) for EtAc extract and ethyl acetate/methanol (9/1,v/v) for MeOH extract.

Spots were visualized by spraying conc.H_2SO_4 and viewing the plates under UV light(254,365 nm) after heating 1 hr at 110°C. Spots inhibiting cholinesterase activity were detected by the following method. The plates were sprayed with an enzyme solution (0.4 mg cholinesterase in 1 ml of Tris-buffer, pH 8.3), and kept at room temperature for 30 min. Then the plate was sprayed with the substrate solution; a mixture of 2.2 ml of 0.02 M Tris-buffer, 0.3 ml of 0.05 M potassium ferrocyanide and ferricyanide solution and 0.85 ml indoxylacetate ethanol solution(3mg/ml). The inhibition areas appeared as white spots on a blue background.

RESULTS AND DISCUSSION

The extract obtained from the river sediment is shown in Table 1. The dose-response curve of cholinesterase (ChE) inhibition of urban or rural river extracts are shown in Figure 3. The inhibitory effect on ChE of each extract was weak. The Hex extracts show the weakest effect. It is apparent that each extract inhibits ChE, because ChE inhibition increases as dose increases for the extracts.

Table 1. Organic matter from river sediment.

Sampling station	n-Hexane (mg/200 g)	Ethyl acetate (mg/200 g)	Methanol (mg/ 200 g)
Urban rivers			
Tsurumi	124	504	176
Meguro	335	145	1057
Sumida	1263	606	102
Ara	544	241	124
Ayase	591	304	118
Rural rivers			
Magame	98	90	90
Sakuta	107	169	64
Kido	563	113	75
Yatsu	64	64	340
Shinbori	837	575	735

Table 2. Inhibition of ChE activity by various solvent extracts.

Sampling station	n-Hexane I_{30}(mg/ml)	Ethyl acetate I_{30}(mg/ml)	Methanol I_{30}(mg/ml)
Urban rivers			
Tsurumi	+	1.51	+
Meguro	-	0.22	+
Sumida	-	0.46	0.63
Ara	+	0.44	0.15
Ayase	+	0.69	+
Rural rivers			
Magame	+	0.43	1.59
Sakuta	-	0.12	+
Kido	-	0.16	1.00
Yatsu	+	+	+
Shinbori	-	0.15	+

(+) below 30% inhibition
(-) negligible inhibition

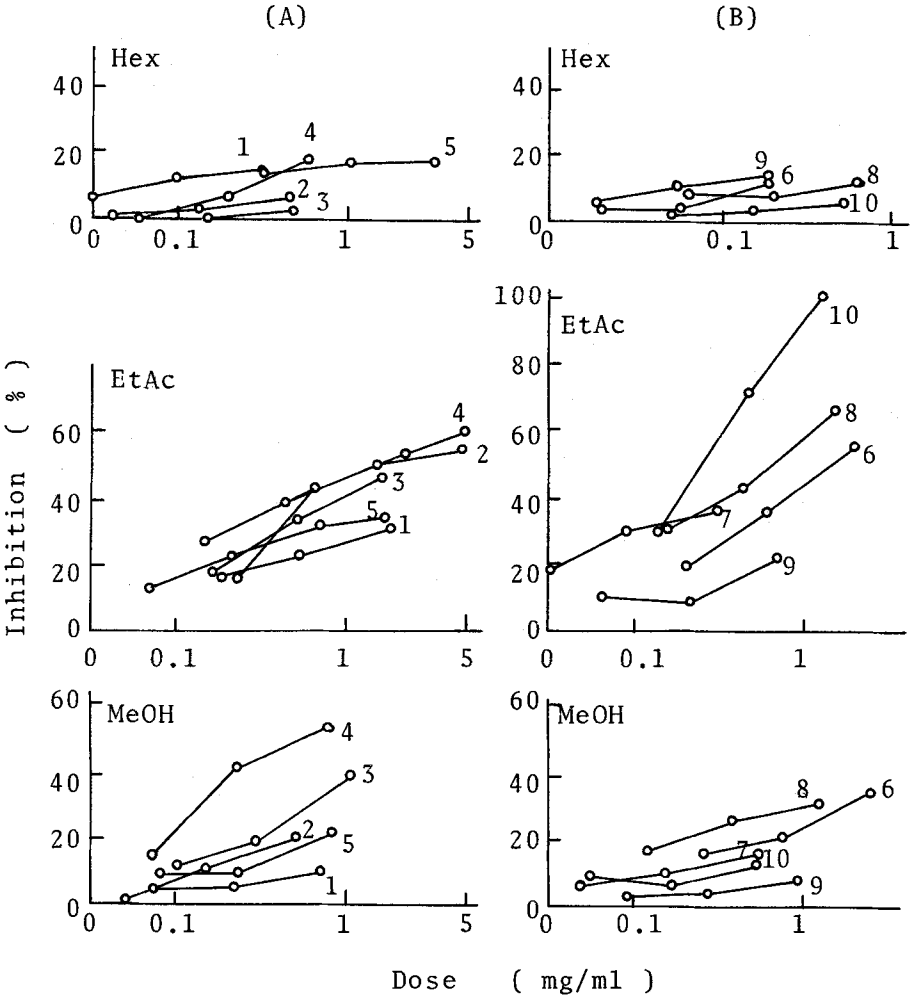

Fig. 3. Cholinesterase inhibition activity from urban(A) and rural(B) rivers.
1. Tsurumi 2. Meguro 3. Sumida 4. Ara 5. Ayase
6. Magame 7. Sakuta 8. Kido 9. Yatsu 10. Shinbori

Table 2 shows the concentration of the extracts with a 30 % level of ChE activity inhibition (I_{30}). The I_{30} Hex extract values were not obtained because ChE inhibition with Hex extracts were too weak. The I_{30} values of EtAc extracts were smaller than the MeOH extracts. Among the urban rivers EtAc extracts, the Meguro and Ara rivers showed strong inhibition, while the Tsurumi river showed the weakest. For MeOH extracts, Ara river extract showed the strongest inhibition. Among the rural rivers, EtAc extracts from the Sakuta and Shinbori

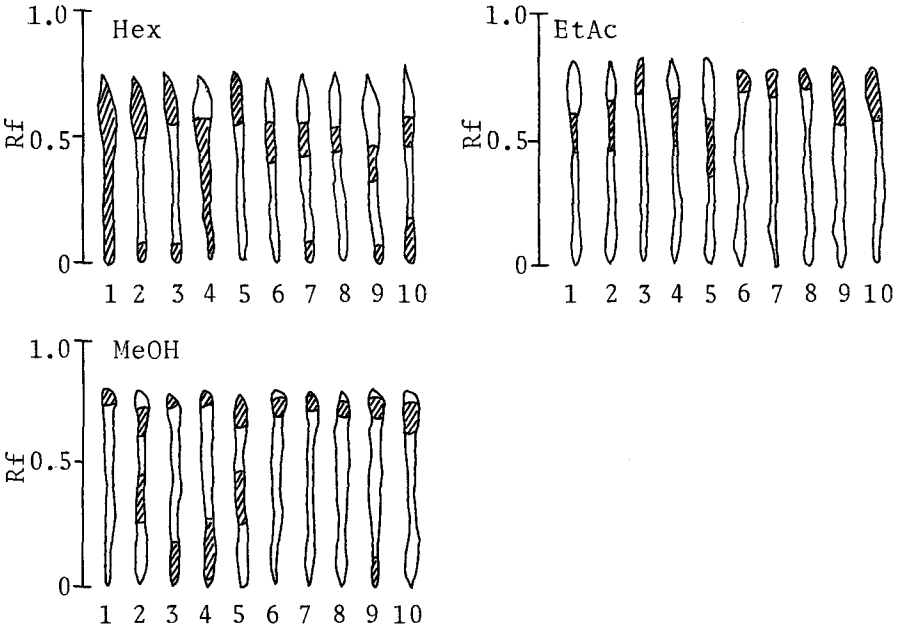

Fig. 4. TLC-separation of solvents extracted organic matters from urban and rural river sediment.
○ organic matter
▨ enzymatic inhibition
urban rivers 1. Tsurumi 2. Meguro 3. Sumida
4. Ara 5. Ayase
rural rivers 6. Magame 7. Sakuta 8. Kido
9. Yatsu 10. Shinbori

rivers showed high inhibition, while the Yatsu river showed less inhibition. MeOH extracts of these rivers showed weak inhibition. Comparing ChE inhibition between the urban and rural rivers indicates that the EtAc extracts of the rural rivers strongly inhibit ChE activity more than the urban rivers. The MeOH extracts from rural rivers inhibited ChE activity less than the urban rivers. The I_{30} values of the extracts ranged from 0.12 mg/ml to 1.59 mg/ml. No great difference was observed in inhibitory effect.

Figure 4 shows the thin-layer chromatography (TLC) pattern of the extracts of the urban and the rural river sediments visualized by the enzyme inhibition technique. Obvious differences in the Rf values of ChE inhibition spots were observed between the urban and the rural rivers. The Hex extracts of the rural river sediments showed spots from Rf 0.05 to 0.1 and 0.5. The urban rivers showed spots from Rf 0.6 to 0.7 or 0.0 to 0.8.

Table 3. I_{30} value for ChE and Rf value on TLC of chemicals

Chemicals	I_{30}(mg/ml)	TLC Rf Hex/EtAc (1/1)	TLC Rf EtAc/MeOH (1/1)	TLC Rf EtAc/MeOH (9/1)
Organophosphates				
diazinon	5.2×10^{-4}	0.55	0.70	0.68
disulfoton	1.2×10^{-3}	0.67	0.71	0.65
fenitrothion	3.2×10^{-3}	0.59	0.71	0.71
malathion	1.8×10^{-2}	0.56	0.72	0.68
o-ethyl o-p-nitrophenyl phenylphosphonothioate	1.2×10^{-1}	0.64	0.75	0.73
Carbamates				
carbaryl	4.8×10^{-3}	0.40	0.67	0.66
3,4-xylyl methylcarbamate	5.7×10^{-3}	0.46	0.68	0.62
propoxur	8.2×10^{-3}	0.42	0.68	0.64
m-tolyl methylcarbamate	1.3×10^{-2}	0.44	0.70	0.64
o-sec-butylphenyl methyl-carbamate	2.5×10^{-2}	0.49	0.71	0.66
3,5-xylyl methylcarbamate	3.0×10^{-2}	0.45	0.70	0.67
Other chemicals				
di-n-butyl phthalate	4.9×10^{-1}	0.63	0.73	0.69
di-2-ethyl-hexyl phthalate	19.5	0.68	0.74	0.71
phenol	0% (9.4×10^{-1})	0.59	0.71	0.66
o-phenyl phenol	6.0×10^{-1}	0.60	0.72	0.70
benzo(a)pyrene	4% (5.0×10^{-5})	0.64	0.70	0.68
polychlorinated biphenyl (KC 500)	1.5	0.68	0.72	0.67
sodium dodecylbenzene sulfate	1.1	0.00	0.60	0.34

The rural river EtAc extracts showed spots at Rf 0.7 to 0.75, while the urban rivers showed spots at Rf 0.5 to 0.6.

For MeOH extracts, ChE inhibition spots were found at Rf 0.8 and 0.2 to 0.45 in the urban rivers, and at Rf 0.0 and 0.75 in the rural ones. No significant difference in ChE inhibition intensity was observed between the urban and the rural river extracts, but there are differences in the Rf value of ChE inhibition spots. It is clear that ChE inhibitors from the urban rivers differ from those from the rural ones.

Table 3 shows I_{30} values and Rf values of organophosphate and carbamate pesticides that are typical ChE inhibitors, and some chemical expected to be present in river sediment. The TLC plates were developed with the same solvents used for sediment extraction. Organophosphates and carbamates inhibited ChE activity much more than the other chemicals. If the inhibitory effect of ChE in river sediment extract is caused by these organophosphate or carbamate pesticides, the intensity of ChE inhibition corresponds by 0.033 to 250 μg to the pesticides per mg of extract. The five organophosphate and six carbamate pesticides resemble Rf values on TLC plates developed with each solvent. Because the ChE inhibition spots of the extracts did not show Rf values similar to the pesticides expect the EtAc extracts of the rural river sediments (Rf 0.7 to 0.75), the ChE inhibition exhibited must be due to substances other than those pesticides. On the other hand, phthalate, o-phenyl phenol, polychlorinated biphenyl, and benzo(a)pyren showed Rf values (Rf 0.6 to 0.7) similar to Hex extracts from the urban river sediment. Hex extracts from the Tama river sediment down stream contained aliphatic, aromatic hydrocabons, organochlorine compounds and phthalate in previous work (Suzuki et al. 1982). It may be suggested that the inhibitory effect of the Hex extracts of the urban rivers is due to the above chemicals. Because of their weak inhibitory effects, these chemicals explain only part of the ChE inhibition, however.

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