Bull. Environ. Contam. Toxicol. (1990) 45:31–38

Bulletin of Environmental Contamination and Toxicology

Evaluation of Pollutant Toxicity in Aquatic Environment by Assay of Enzymes Released from Lysosomes

Masako Tabata, Yoshikazu Kobayashi, Atsushi Nakajima, and Shizuo Suzuki

Faculty of Pharmaceutical Science, Science University of Tokyo,
12 Funagawara-machi, Ichigaya Shinjukuku, Tokyo 162, Japan

To survey aquatic environmental pollution, many workers have attempted to evaluate river pollution using index organisms. These methods reflect the toxicities of river water and sediment directly. In recent years, the monitoring method using enzyme inducement or enzyme depression in fish or other aquatic organisms has been proposed for studying polluted environments (Payne and Penrose 1975; Kurelec et al. 1977; Verma et al. 1979; Wieser and Hinterleitner 1980). However those methods cannot evaluate toxicity easily because of individual difference of test organisms or difficulty of capture of organisms. To evaluate toxicity of environmental sample simply, we attempted to use biochemical index for assay method.

Lysosomes are present in the endoplasmic reticulum enclosed by a membrane and contain several kinds of hydrolases such as acid phosphatase and β-glucuronidase. When the membrane of a lysosome is destabilized by chemical action, resident enzymes are released (Dean 1981). The effect of chemicals on a lysosome membrane thus can be evaluated by measuring the activity of released enzymes. In the present paper we evaluate environmental sample toxicity for biological membrane using rat liver lysosomes in vitro.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 150-180 g were used. They were fasted for 24 hr before sacrifice and the livers were immediately perfused with ice cold 0.25 M sucrose solution (pH 7) through the hepatic portal vein using a syringe and then excised. After being weighed each liver was minced and homogenized with 0.25 M sucrose solution (1 mL/g wet liver) by a Waring blender (Ace homogenizer AM-4 Japan) for 30 sec. After adding a

---

Send reprint requests to M. Tabata at the above address.

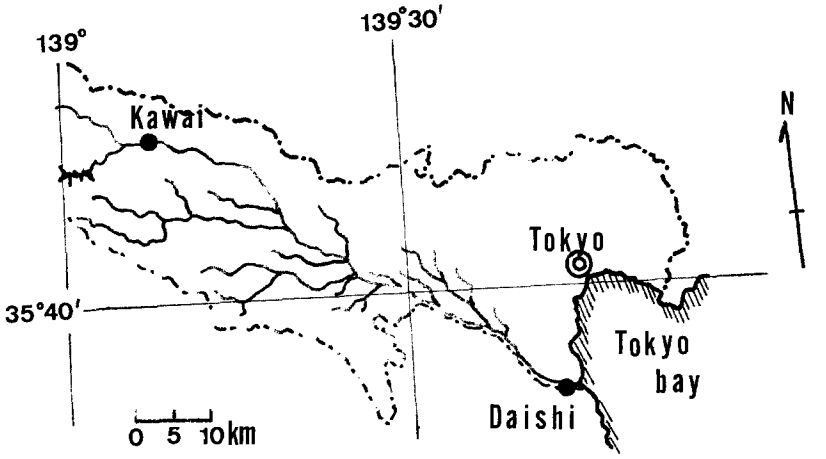

Figure 1. Sampling sites of the Tama river.

double volume of 0.25 M sucrose solution, homogenation was repeated with a teflon Potter Elvehjem homogenizer. The solution was fractionated by centrifugation according to the method of Takahashi et al.(1979) and obtained the lysosome fraction. Lysosome precipitants were suspended in a 0.7 M sucrose solution (10 mL/g wet liver). The lysosome suspension was stored at -80°C subdividing in small vessels in order to suffer no freez/thaw cycle on a assay. Protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Thirty-eight kinds of test chemicals of precision analytical grade were dissolved in water or methanol. Sediment samples were collected from the surface layer (0-15 cm) using a shovel or an Ekman-Birge dredge (Rigoh-sha Japan) at the upper and lower reaches of the Tama River on 6 June, 1983, as shown in Figure 1. Each 200g of the dried sediment were extracted successively every 8 hours with n-hexane and methanol as reported previously (Tabata et al. 1984).

The release of acid phosphatase from the lysosomes was measured as an indicator of instability of the lysosome membrane. A mixture of 1.8 mL of lysosome suspension (0.02 mg protein/mL 0.7 M sucrose solution) and 0.2 mL of test chemical solution was incubated for 30 min at 37° C, followed by centrifugation at 9500 g at 20 min at 0° C. The acid phosphatase activity of the supernatant was measured. This is called soluble acid phosphatase activity. Total acid phosphatase activity of lysosome was measured for 1.8 mL of lysosome suspension with 0.2 mL of 1% Triton X-100 as same as above.

Acid phosphatase activity was measured as follows: A mixture of 1 mL of citric acid buffer(0.1 M citric acid/

0.1 M sodium citrate/H_2O 23/27/50 pH 4.8), 0.25 mL of 0.055 M sodium p-nitrophenyl phosphate dissolved in citric acid buffer and 1 mL of the supernatant of the centrifuged lysosome suspension was incubated for 30 min at 37°C. The mixture without sample or substrate were also incubated as control. One mL of 0.5 N NaOH solution was then added to stop the reaction. Absorption of the solution at 410 nm was measured using a spectrophotometer (Hitachi 101).

RESULTS AND DISCUSSION

The effects of solvents in dissolved samples on the acid phosphatase (Acid PA) activity and the release rate were examined (Table 1). Total and soluble Acid PA activities of lysosome suspension with 10% of water, dimethylsulfoxied (DMSO), methanol, n-butyl acetate or DMSO/n-butyl acetate (4/1) solution were measured. Total Acid PA activity of lysosome suspension with each solvent was expressed as the percent against the activity with water. Acid PA release rate was expressed as the percent of soluble Acid PA activity to total activity of lysosome suspension with each solvent. n-Butyl acetate strongly inhibited total Acid PA activity. The released rate was less affected by DMSO or methanol. Considering the solubility of chemicals or the sediment extract and the affinity of the solvents with lysosome suspension, the test chemicals had been dissolved in water or methanol and the sediment extracts in mixture of DMSO/n-butyl acetate (4/1) solution.

The action of 38 chemicals possibly present in a aquatic environment on a lysosome membrane was tested according to the method described above. Figure 2 shows the dose response curve of benzothiophene as a representative case. Figure 2-A shows total and soluble Acid PA activity for each concentration of benzothiophene in lysosome suspensions. Figure 2-B shows the inhibition percent of total Acid PA activity and the actual release rate of enzyme from lysosome on various concentration. Enzyme inhibition and actual release rate were estimated by the following equations. To measure the Acid PA activity of blank, each solvent was added to the lysosome suspension instead of sample solution.

$$\text{Enzyme inhibition (\%)} = 1 - \frac{\text{total Acid PA activity of sample}}{\text{total Acid PA activity of blank}} \times 100 \quad \text{(I)}$$

$$\text{Actual release rate (\%)} = \frac{\left(\text{soluble Acid PA activity of sample}\right) - \left(\text{soluble Acid PA activity of blank}\right)}{\left(\text{total Acid PA activity of sample}\right) - \left(\text{soluble Acid PA activity of blank}\right)} \times 100 \quad \text{(II)}$$

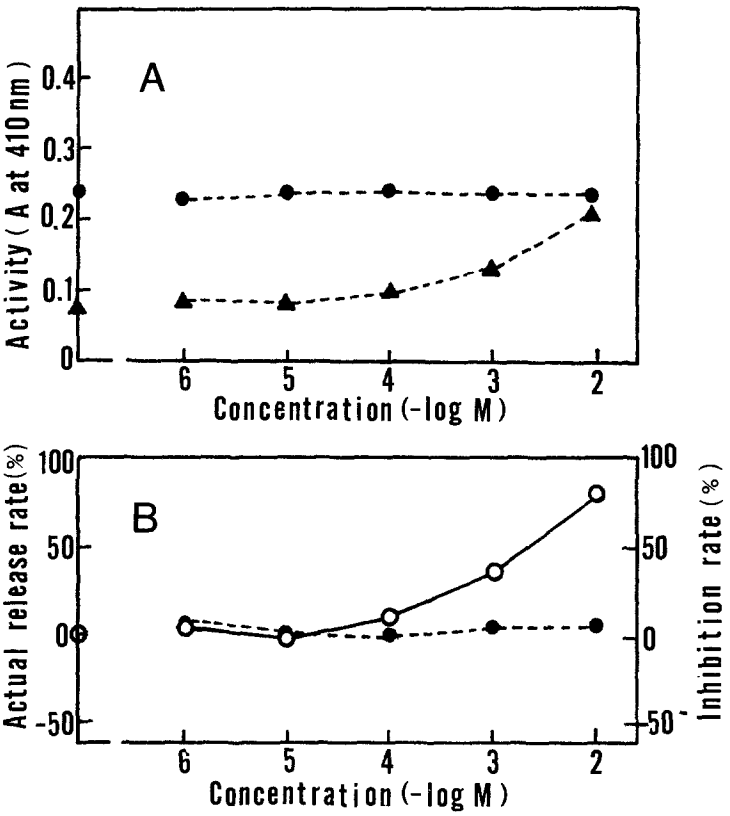

Figure 2. Effect of benzothiophene on rat liver lysosome. Total activity of acid-phosphatase (A-●), soluble activity of acid-phosphatase (A-▲); actual release rate of acid-phosphatase (B-○) and inhibition rate of acid-phosphatase (B-●).

Table 1. Effects of solvents on the acid phosphatase activity and the release rate of the enzyme from lysosome.

Solvent[1)]	Total acid phosphatase activity of lysosomes (%)	Acid phosphatase release rate from lysosome[2)] (%)
H_2O	100.0	39.1
DMSO	98.9	51.8
methanol	85.8	62.3
n-butyl acetate	29.2	75.6
DMSO/n-butyl acetate (4/1)	60.7	69.5

1) 10 % In preincubation lysosome suspension.
2) Soluble acid phosphatase activity x 100
Total acid phosphatase activity

Table 2. Effects of chemicals on rat liver lysosome

Chemicals	Actual release rate of acid-phosphatase (%)					Acid-phosphatase inhibition
	Dose (mol/L)					
	$1x10^{-6}$	$1x10^{-5}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-2}$	ID_{50} (mol/L)
NAC	NR[a]	NR	NR	12	-[b]	NI[c]
DDVP	NR	NR	35	51	-	NI
Sumithione	NR	NR	20	NR	-	NI
PCP	NR	22	18	-	-	NI
DDT	NR	25	20	-	-	NI
Aldrin	NR	32	14	NR	-	NI
γ-BHC	NR	NR	31	30	-	NI
Triphenyltin acetate	NR	NR	-	-	-	NI
Sodium dodecyl-benzenesulfate	NR	NR	50	-28	-	$1x10^{-3}$
Benzalkoniumchloride	NR	50	40	85	-	$1x10^{-3}$
Tween 60	NR	NR	36	60	-	NI
Palmitic acid	NR	41	38	37	-	NI
Oleic acid	NR	38	22	19	-	NI
Di-n-butyl phthalate	NR	NR	30	-	-	NI
Benzene	NR	NR	NR	NR	NR	NI
Benzo(a)pyrene	NR	NR	-	-	-	NI
Dodecylbenzene	NR	NR	NR	NR	-	NI
Chloroform	NR	NR	NR	NR	NR	NI
Trichloroacetic acid	NR	NR	15	95	-	$1x10^{-3}$
Chlorobenzene	NR	NR	NR	12	NR	NI
2,4,6-Trichlorophenol	NR	NR	11	30	-	NI
PCB	NR[d]	24[e]	21[f]	-	-	NI
Phenol	NR	NR	NR	NR	28	NI
α-Naphthol	NR	NR	NR	22	-	NI
Resorcinol	NR	NR	NR	NR	-	NI
o-Phenylphenol	NR	NR	22	35	-	NI
Benzophenone	NR	NR	NR	41	-	NI
Benzothiophen	NR	NR	10	36	80	NI
Nitrobenzene	NR	-12	NR	32	12	NI
Nitrofluorene	NR	NR	12	-	-	NI
Dimethylaminoazo-benzene	NR	NR	-	-	-	NI
Nitrilotriacetic acid	NR	20	-	-	-	NI
Phenylhydrazine	45	80	54	35	-	NI
2,4;6-trinitrophenol	NR	19	NR	-	-	NI

Table 2 continued.

Chemicals	Actual release rate of acid-phosphatase (%)					Acid-phosphatase inhibition
	Dose (mol/L)					
	$1x10^{-6}$	$1x10^{-5}$	$1x10^{-4}$	$1x10^{-3}$	$1x10^{-2}$	ID_{50} (mol/L)
Aniline	NR	NR	NR	12	12	NI
α-Naphthylamine	NR	-11	-13	NR	-	NI
Dimethylnitrosamine	NR	NR	NR	-	-	NI
Pyridine	NR	NR	-15	-11	-20	NI

a) NR means that the release of acid-phosphatase was negligible (less than 10%).
b) At this dose, assay was not done.
c) NI means that the inhibition of acid-phosphatase was negligible.
d) The dose of PCB was 10 ppm.
e) The dose of PCB was 100 ppm.
f) The dose of PCB was 1000 ppm.
NAC: 1-Naphthyl-N-methyl-carbamate
DDVP: o,o-Dimethyl-o-(2,2-dichlorovinyl) phosphate
Sumithione: o,o-Dimethyl-o-4-nitro-m-tolyl phosphorothioate
PCP: Pentachlorophenol
DDT: 2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane
Ardrin: 1,2,3,4,10,10-hexachloro-1,4,4a5 8 8a-hexahydro-endo-exo-1,4:5,8-dimethanonaphthalene
γ-BHC: Benzenehexachlpride
PCB: Poly chlorinated biphenyl (KC 500)

The actual release rate and dose of 50 % inhibition on total Acid PA activity about 38 chemicals are shown in Table 2.

On evaluation of enzyme release effect of sample, inhibition effect for Acid PA should be considered also. The actual release rate increased for surfactants used, $>10^{-6}$ M for benzalkonium chloride and $>10^{-5}$ M for sodium dodecyl benzensulfate and Tween 60. Sodium dodecyl benzensulfate also inhibited Acid PA activity. Surfactants destroy the hydrophilic bonds between lipids and proteins in the membranes of organisms, and of course in this study, they destabilized the lysosome membrane.

Trichloroacetic acid, used as a denaturant of protein, inhibited enzyme activity at lower concentration than other chemicals and released Acid PA 10^{-4} M concentration. Amines, aromatic hydrocarbons and phenol compounds were ineffective. Organochlorine compounds

Table 3. Effects of sediment extracts on rat liver lysosome

Extracts	Dose (mg/mL)	Actual release rate of enzyme[1] (%)	Inhibition rate of enzyme[2] (%)
Kawai			
n-Hex extract	0.05	20.6	2
	0.5	20.3	-1
MeOH extract	0.05	-20.5	-2
	0.5	72.2	0
Daishi			
n-Hex extract	0.05	-1.8	-1
	0.5	56.7	-2
MeOH	0.05	9.5	0
	0.5	103.6	-6

1) Calculated from equation (II)
2) Calculated from equation (I)

showed weak destabilizing effect. Benzothiophen, which attacks the mitochondorial membrane of rat liver to release potassium ions (Ogata and Hasegawa, 1982), exhibited strongly effect. Fatty acid, oleic acid and palmitic acid destabilized the lysosome membrane, but the mechanisms involved are obscure.

As far as the present investigations are concerned, the instability of lysosome membranes is not caused by special chemicals, but by many factors such as binding ability to protein, which may be involved in the disruption of lysosome membranes, or attack to the unsaturated fatty acids of membranes to form lipid peroxides (Desai et al. 1964, Nakanishi et al. 1975). The assay method using lysosomes makes possible to monitor the effect of environmental pollutants on biomembranes. But the lysosome membrane destabilizing effects are not specific to certain chemicals, thus identification of pollutants in natural samples may be difficult by this assay.

Our assay method was applied to sediment samples of the Tama River to evaluate the toxicity of the samples. The sampling site, Kawai, is situated in upper reaches of the Tama River and little polluted by the waste water. Daishi is situated about 1 km upstream from the river mouth where the river is usually polluted by domestic and industrial waste water.

Table 3 shows the actual release rate of Acid PA from lysosomes and inhibition rate of total Acid PA activity in sediment sample assay. The methanol extracts of Kawai and Daishi sediments showed 72.2 % and 103.6 % of

actual release rate at 0.5 mg/L, respectively. Hexane extract of Dishi sediment showed 56.7 % at 0.5 mg/L. These results indicate that destabilizing substances of lysosome membranes are present in the river sediments. It is noticeable that the lower reach sediment extracts, where is supposed accumulation many kinds of pollutants, showed stronger effect on lysosomes than the upper reach sediment extracts.

Acknowledgments. This study was supported by a grant in-aid for a special project on environmental science under grant No. 58030066 from the Ministry of Education Science and Culture.

REFERENCES

Dean RT (1981) Lysosome. Asakura-shoten, Tokyo pp90

Desai ID, Sawant PL and Tappel AL (1964) Peroxidative and radiation damage to isolated lysosomes. Biochem Biophys Acta 86:277-285

Kurelec B, Britvic S, Rijavec M, Muller WEG and Zahn RK (1977) Benzo(a)pyren monooxygenase induction in marine fish molecular response to oil pollution. Mar Biol 44:211-216

Lowry OH, Rosebrough NJ, Farr AL and Randoll RJ (1951) Protein measurement with the folin-phenol reagent. J Biol Chem 193:265-275

Nakanishi M, Goto K and Hisadome M (1975) Studies on anti-inflammatory agents XXIX. Inhibitory effects of Tinoridine on release of lysosome enzymes and lipid peroxidation in rat liver. Yakugaku Zasshi 95:936-943

Ogata M and Hasegawa R (1982) The effects of benzothiophene and dibenzothiophene on the mitochondrial membrane of the goldfish. Igaku to Seibutsugaku 104:247-250

Payne JF and Penrose WR (1975) Induction of aryl hydrocarbon (Benzo(a)pyrene) hydroxylase infish by petroleum. Bull Environ Contam Toxicol 14:112-117

Tabata M, Bannai E, Nishizono H and Suzuki S (1984) In vitro cholinesterase inhibition of organic matter in urban and rural river sediment. Bull Environ Contam Toxicol 32:391-399

Takahashi K, Saeki S and Kato K (1979) Effect of polychlorinated biphenyls on rat liver lysosomal membranes. Fukuoka Igaku Zasshi 70:93-98

Verma SR, Tyagi AK and Dalela RC (1979) Effect of distillery waste on some freshwater teleosts biochemical studies. Environ Pollut 19:225-228

Wieser W and Hinterleitner (1980) Serum enzymes in rainbow trout as tools in the diagnosis of water quality. Bull Environ Contam Toxicol 25:188-193

Received July 5, 1989; accepted December 18,1989.