Evaluation of Interaction of Paraquat with Lecithin or Casein by Paramecium

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A paramecium was employed as a biological reagent to evaluate the interaction of paraquat with biological materials (additives). This protista was added into a cocktail of paraquat and a biological material preparation. Life or death of paramecium in the cocktail indicated whether or not paraquat interacted with an additive. The following materials were found to protect paramecia from the toxicity of paraquat: soybean lecithin, soybean milk, egg lecithin, casein, cow's milk, skim milk, cheese, and trypan blue (not a biological material). These findings suggested that those materials had an ability to interact with paraquat.

Keywords — paraquat; lecithin; casein; trypan blue; interaction; paramecium

Introduction

Paraquat (1,1'-dimethyl-4,4'-bipyridylium dichloride) has been reported to exert its toxicity at the following steps of a mechanism: one electron reduction of paraquat, generation of active oxygens, oxidation of biologically high molecular materials, and generation of peroxidants.¹⁻³⁾ Other than this mechanism, however, the direct toxic interaction of paraquat with biological tissue materials was considered at an early stage of exposure as well as the cell membrane toxicity of other quaternary bases: benzalkonium chloride, benzethonium chloride, *etc.* Our previous paper⁴⁾ monitored different tissue samples capable of interacting with paraquat by thin-layer chromatography on a wet Sephadex G-25 plate. A rat brain whole homogenate sample showed a significant affinity for paraquat. Many delipidized tissue samples (water-insoluble) of rat were bound with paraquat, based on a principle of ion exchange reaction. The present work further attempted to determine the chemical species of the biological samples used. Equilibrated

interaction between a biological material and paraquat is not observable by electrophoresis, dialysis, chromatography, or other means, because the interaction is released by electrostatic force or dissociation in a large amount of medium. This problem was thought to be eliminated by employing a paramecium as a biological reagent using the following concept: if interaction between a tissue sample (additive) and paraquat is positive, the paramecium added later will not be affected by paraquat, that is, it will be kept alive; if the interaction is negative, the paramecium added later will die. This concept is supportable, because physiological actions of the following medicines are among those repressed by binding with plasma albumine: warfarin, dicumarol, tolbutamide, phenylbutazon, and indomethacin. A paramecium was cultured in a mixture of paraquat and a certain material (additive). Results of the present study showed that biological materials or foods protected the paramecium from the paraquat toxicity: soybean lecithin,⁵⁻⁶⁾ soybean milk, egg lecithin, cow's milk, cheese, casein, and trypan blue (not a biological material). The

phosphono group was suggested to contribute to the interaction with paraquat. In this study, one of the preparations of paraquat (Gramoxone[®]: paraquat dichloride 24 w/w%) was used as a source of paraquat in conformity with actual toxic phenomena which are caused by erroneous ingestion of the paraquat preparations. It was confirmed in a preliminary experiment that pure paraquat itself similarly killed paramecia under the same experimental conditions as those used in the present study.

Experimental

Chemicals — Paraquat; Gramoxone 100[®] (paraquat dichloride 24 w/w%, d_4^{20} 1.07) was purchased from Takeda Chemical Industries, Ltd. (Osaka, Japan). This was diluted 1000-fold with water for a working solution (paraquat dichloride 0.25 $\mu\text{g}/\mu\text{l}$). Soybean lecithin, soybean oil, egg lecithin, egg albumin, casein, Chondroitin sulfate-Na (a special grade), chitin, trypan blue (direct blue 14), amaranth, and methylene blue were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Centrolux lecithin[®] (95–98% phospholipid content) was purchased from Central Soya, Ltd. Trypan red (CI-22850), direct blue (direct blue 2B), and Evans blue (direct blue 53) were purchased from Tokyo Kasei Industries Co., Ltd. Cow's milk: Meiji 3.6 MILK[®] was purchased from Meiji Lacto Industries, Ltd. Skim milk: Skim plus[®] (30% protein content) and CREAMA[®] were purchased from Yukijirushi Lacto Industries, Ltd. Butter (natural) and cheese (process slice type) were also purchased from Yukijirushi Lacto Industries, Ltd. Soybean milk: Prepared Soybean Milk[®] (plant protein 3.6% and lipid 3.85% contents) was purchased from Kibun Food Chemipha, Ltd. Salmon testis DNA-Na, type III, was purchased from SIGMA[®] Chemical Company. Animals: Wistar strain male rats (250 g) and Japanese White strain female rabbits (4 kg) were purchased from Sankyo Labo Service Co. Paramecium (0.13 mm body length) culture solution: a kind of protist collected from a pond was cultured in a medium of filtered pond water (10

–20 ml) containing one grain of rice in a petri dish (5 cm ϕ) at room temperature.

Evaluation of Interaction of Paraquat with Additive — Paraquat solution (paraquat dichloride 0.25 $\mu\text{g}/\mu\text{l}$, 20 μl) was mixed with an additive (50 μl) in a 5 cm ϕ disposable plastic petri dish. This primary mixture is referred to as a cocktail in the remainder of experiment. A paramecium culture solution (50 μl , containing 10–20 paramecia) was added to the cocktail to be mixed. The mixture in the dish was spread in a circle to a size of 1.5 cm ϕ and observed under an Olympus (CK-2 microscope ($\times$ 75–150).

Drying Method for Sample Preparation — Tissue sample solution (less than 50 ml) was spread on a glass plate (20 $\times$ 20 cm) and left standing overnight at room temperature. The dried material stuck on the plate was scraped off using a single-edge safety razor (Feather-S[®]).

Preparation of Tissue Additive — A rabbit was sacrificed by injecting air into the ear vein. Each tissue excised was washed with water to remove excess blood, and homogenized with one portion of water to give a whole homogenate using a Yamato Labo-Stirrer, model LA-41B. This was called additive W. A part of the homogenate was dried and extracted with chloroform. After evaporation of the solvent, the oil residue was emulsified in water using a Branson Sonifier 250 sonicator to give additive L (10 mg/ml). The water insoluble material (remainder after extraction) was powdered and suspended in water to give additive P (10 mg/ml). A rat was sacrificed by decapitation, and similarly treated as above to give additives W, L, and P.

Preparation of Treated Lecithin Additive — Soybean lecithin (0.5 g) was shaken in a 1.6 mm i.d. $\times$ 18 cm long test tube with 10 ml of 0.1 M potassium phosphate buffer solution of differing pH as indicated in the abscissa of Fig. 2. The mixture was left standing overnight at room temperature. The lecithin layer separated from the aqueous layer was taken onto a filter paper to remove excess water. The treated lecithin (100 mg) was shaken in a 0.9 mm i.d. $\times$ 5 cm long

cappable glass tube with paraquat solution (paraquat dichloride $0.25 \mu\text{g}/\mu\text{l}$, 1 ml). The aqueous layer separated from the lecithin was subjected to electrophoresis.

Electrophoresis for Determination of Paraquat — A paraquat solution ($10 \mu\text{l}$) obtained after shaking with the treated lecithin was spotted along a center of a 18 cm wide $\times$ 20 long (working area) Toyo Roshi filter paper (No. 526), which was previously dampened with 0.1 M $\text{K}_2\text{HPO}_4\text{-NaOH}$ solution (pH 12). This was electrophoresed under the following conditions: both electrode solutions, the same buffer as above; 100 V constant; 35–45 mA; and for 1 h at room temperature. Thereafter, the paper was dried and heated at 100°C in an oven until the appearance of a blue spot of paraquat. The blue spot was read on a Shimadzu CS-910 densitometer (Kyoto) under the following conditions: absorbance, 625 nm; slit size, $1 \text{ mm} \times 10 \text{ mm}$; plate and chart speeds, 80 mm/min; and recorder sensitivity, $\times 10$. The chart obtained was copied onto another paper, and peaks on the copy were cut out

to be weighed to determine the amount of paraquat.

Results

A cocktail of paraquat alone killed all paramecia used through the stages of body swelling, body puncturing, and scattering of particulate contents as shown in Fig. 1. Table I-1 summarizes results of the evaluation of the life and death of paramecia, which were cultured in cocktails of paraquat and various rabbit tissual additives. The first column of Table I-1 suggests that additives W of brain, vitreous body, crystalline lens, harderian gland, lung, heart, liver, and kidney were able to interact with paraquat. Particularly, additives W of brain and lung appeared to bind with methylene blue (MB: quaternary ammonium ion) too, which was toxic to paramecium (fourth column of Table I-1). Additives W of stomach, small intestine, and large intestine alone killed paramecia in spite of the absence of paraquat (first column of Table

Fig. 1. Profiles of Death of Paramecium Damaged by Paraquat or Methylene Blue

A: normal paramecium. B: the primary step of damage, swelling accompanied by uneven distribution of particulate contents. C and D: puncturing accompanied by scattering of particulate contents.

TABLE I-1. Effects of Additives of Rabbit Tissue Preparations on Toxicity of Paraquat to Paramecium

Additive of W, P, and L from tissue samples	PQ +	PQ +	PQ +	MB +
	W	P	L	W
Brain	+	+	+	+
Vitreous body	+	+	±/-	-
Crystalline lens	+	+	±	±
Harderian gland	+	+	±/-	±/-
Lung	+	+	±	+
Heart	+	+	±/-	±/-
Liver	+	+	±/-	-
Kidney	+	+	±/-	±
Adrenal	±	±	±/-	±
Spleen	±	+	±/-	±/-
Stomach ^{a)}	-	±	±/-	-
Small intestine ^{a)}	-	±	±/-	-
Cecum ^{a)}	-	±	±/-	-
Colons ^{a)}	-	±	±/-	-
Appendix ^{a)}	-	±	±/-	-
Bile juice ^{a)}	-	-	-	-
Water (control)	-	-	-	-

Mixing method: a paramecium (0.13 mm body length) culture solution (50 μ l involving 10–20 paramecia) was mixed with a cocktail of paraquat (PQ-2Cl, 0.25 μ g/ μ l, 20 μ l), or methylene blue (MB, 2.5 μ g/ μ l, 10 μ l), and additive solution (50 μ l) of W, P, or L. Additive W: tissue whole homogenate of a 4-kg female rabbit (1:1, w/v in water). Additive L: chloroform extract (oil) of dried additive W, 10 mg/ml in water, emulsion form. Additive L: water-insoluble material remaining after chloroform extraction, 10 mg/ml, suspension form. Evaluation: -, all paramecia used died immediately after mixing; ±, all paramecia used died within 0.5–2 h after mixing; and +, at least a few (10–20%) of paramecia used lived for more than 8 h. Mark /: no examination. Mark a): paramecia used all died in the presence of this additive W alone.

I-1). The death of paramecia implied that these tissual additives W had some component(s) which deteriorated paramecium. Additives P of stomach, small intestine, and large intestine, however, were suggested to have a somewhat intrinsic ability to interact with paraquat (second column of Table I-1). Among various additives L, that of brain showed a characteristic ability to interact with paraquat differing from the other tissual additives L (third column of Table I-1).

Table I-2 summarizes results obtained using rat tissual additives. The same evalua-

TABLE I-2. Effects of Additives of Rat Tissue Preparations on Toxicity of Paraquat to Paramecium

Additive of W, P, and L from tissue samples	PQ +	PQ +	PQ +	MB +
	W	P	L	W
Brain	+	+	+	+
Eyeball	+	+	-	-
Harderian gland	+	+	-	±
Submaxillary gland	+	+	-	±
Lung	+	+	-	-
Heart	+	+	-	+
Liver	+	+	-	-
Kidney	+	+	-	-
Adrenal	±	±	-	-
Spleen ^{a)}	-	±	-	-
Pancreas ^{a)}	-	±	-	-
Stomach ^{a)}	-	±	-	-
Small intestine ^{a)}	-	±	-	-
Large intestine ^{a)}	-	±	-	-
Seminal vesicle	±	±	-	-
Prostate	+	+	-	±
Testis	+	+	-	-
Water (control)	-	-	-	-

Mixing method and evaluation were the same as in the legend to Table I-1 except for the change from rabbit to rat.

tions were done for each additive preparation as done for rabbit. Additives P of stomach, small intestine, and large intestine were suggested to have a somewhat intrinsic ability to interact with paraquat (second column of Table I-2). Additive L of brain also showed a characteristic ability to interact with paraquat (third column of Table I-2). These findings were identical to those of rabbit.

The interaction of paraquat with brain fatty material was thought to be based on an interaction with phospholipids, because the brain contains a large amount of these materials. Table II summarizes the evaluation of life and death of paramecia cultured in cocktails of paraquat and various emulsions of lecithins or other materials. The evaluations suggested that lecithins or lecithin-rich food materials were able to interact with paraquat as shown in the first column of Table II. The phosphono group in the structure of lecithin was speculated to contribute to the interac-

TABLE. II. Effects of Additives of Foods on Toxicity of Paraquat to Paramecium

Aqueous solution (S) of additive	PQ	MB
	+	+
	S	S
Soybean lecithin (emulsion: 100 mg/ml)	+	+
Centrox lecithin [®] (emulsion: 100 mg/ml)	+	+
Soybean oil (suspension: 100 mg/ml)	±	—
Soybean milk (food and drink)	+	+
Egg lecithin (emulsion: 100 mg/ml)	+	+
Egg albumin (solution: 100 mg/ml)	±	±
Cow milk (food and drink)	+	+
Skim milk ^{a)} (solution, 100 mg/ml)	+	+
Cow milk casein ^{b)} (solution, 100 mg/ml)	+	+
Cheese ^{c)} (emulsion: 100 mg/ml)	+	+
Natural butter ^{d)} (suspension: 100 mg/ml)	±	—
Chondroitinsulfate-Na (solution: 100 mg/ml)	±	±
Chitin (suspension: 100 mg/ml)	±	±
Salmon testis DNA-Na (solution: 20 mg/ml)	±	±
Trypan blue ^{e)} (10 mg/ml)	+	/
Trypan red ^{f)} (10 mg/ml)	—	/
Direct blue ^{g)} (10 mg/ml)	—	/
Amalanth ^{h)} (10 mg/ml)	—	/
Evans blue ⁱ⁾ (10 mg/ml)	—	/
Water (control)	—	—

Mixing method and evaluation were the same as in the legend to Table I-1 except that the MB (methylene blue) added was 10 μ l rather than 20 μ l. *a)*: treated powder obtained by dialyzing an aqueous solution of this food against water and drying. *b)*: treated powder obtained by dialyzing an NH_4OH alkaline solution of this material against water and drying. *c)*: solid type. *e)*: effective at pH 7–8. *f)*: paramecia used all died in the presence of this dye alone in spite of the adjustment of pH.

tion with paraquat. Casein was recognized as another phosphono group-rich material. Casein or casein-rich food materials is also suggested to have the ability to interact with paraquat (the first column of Table II). Albumin (egg) and triglycerides (soybean oil) showed poor ability to interact with paraquat, as expected. Lecithin, lecithin-rich materials, casein, and casein-rich materials had a concurrent ability to bind with methylene blue as shown in the second column of Table II. As other basic dyes, methylgreen and pyronine showed the same binding ability as methylene blue (data not shown). In connection with the phosphono group, some sulfonic group-rich materials were examined. Trypan blue was

found to have the ability to interact with paraquat at a pH of 7–8, but not at an acidic pH.

The interaction of paraquat with lecithin was otherwise confirmed without using paramecium (Fig. 2). After shaking soybean lecithin with different pH buffer solutions, the low pH buffer solutions shifted upward in pH from the theoretical curve. Additional shaking of the treated lecithins with paraquat solution left most of paraquat in the aqueous layer. These findings were interpreted as follows: the choline group of lecithin worked to neutralize the acidic buffers, and was itself ionized. The phosphono group was not ionized and consequently failed to exert an ability to bind with paraquat; on the contrary, the high pH buffer solutions shifted downward in pH from the theoretical curve. Additional shaking of the treated lecithins with paraquat solution removed most of paraquat from the aqueous phase. Thus, the phosphono group worked to neutralize the basic buffers, and was itself ionized to exert its ability to bind with paraquat.

Discussion

The present work employed the phenomenon of life and death of paramecium, as a biological reagent, to evaluate some materials (called additives) which are capable of interacting with paraquat. The concept is as follows: The life of paramecium means a closer interaction of paraquat with an additive than with paramecium. The death of paramecium means a greater familiarity of paraquat with paramecium than with an additive. Naturally, a deteriorative additive contradicts this concept, because paramecia die in spite of the presence or absence of paraquat. Before evaluating an additive, it is necessary to check whether or not it is harmless to paramecia by using a cocktail of the additive alone. Paramecium, however, answered this purpose, because it sensitively responds to environmental chemicals, and the response is

Fig. 2. Adsorption of Paraquat to Lecithin (Soybean)

Bottom panel, $\odot$ — $\odot$: pH variation of original buffer solutions (0.1 M, potassium phosphate) after shaking each buffer solution (10 ml, pH indicated in abscissa) with a soybean lecithin (0.5 g). Bottom panel, -----: theoretical pH curve of original buffer solutions. Top panel, $\odot$ — $\odot$: the ratio of paraquat remaining in aqueous phase to original paraquat added in aqueous phase after shaking a paraquat solution (paraquat dichloride 0.25 $\mu\text{g}/\mu\text{l}$, 1 ml) with a treated lecithin (100 mg), which was obtained by the operation described in the legend of the bottom panel.

recognized as movement. A previous paper⁴⁾ demonstrated that delipidized tissue materials of rat (water-insoluble) were bound with paraquat by ion exchange reaction. Lecithin (soybean) was also demonstrated to be bound with paraquat by ion exchange reaction (Fig. 2). Methylene blue was bound by casein or lecithin, each dyed blue (Table II). These kinds of materials are the main constituents of cell membrane. Therefore, the immediate death of paramecium after interaction between it and paraquat was speculated as being caused by ionic binding of paraquat (cation) with membrane (anion) of paramecium, because paramecium dyed with methylene blue died, but it did not die with acidic dyes (data not shown). This ionic binding itself might result in destruction of the membrane of paramecium, which might lead to the death of paramecium (Fig. 1), as in the case of cell membrane destruction by

other quaternary bases: benzalkonium chloride, benzethonium chloride, *etc.* Paramecia were cultured in a cocktail of paraquat and different materials (additives). Results showed (Table II) that lecithin (phospholipid), casein (phosphoprotein), and trypan blue (one of the 2,7-disulfanaphthalene derivatives)⁷⁾ preferably interact with paraquat (alkylbipyridylum ion), that is, paramecia cultured in the cocktail were kept alive. The phosphono group involved in these chemicals except trypan blue was suggested to ionically bind with paraquat (Fig. 2). Low interaction of paraquat with additive P preparations of digestive organs (Tables I-1 and I-2) was interpreted as follows: the phosphono group might not be strongly involved in the high molecular constituents of these tissues. High interaction of paraquat with additive L preparations of brains (rat and rabbit) was interpreted as follows: the brain tissue

has a large quantity of phospholipid. The affinity of lecithin with paraquat implied the possibility of the binding of paraquat with cellular membrane phospholipids. This binding will necessarily result in the destruction of membrane and the death of cells. The paramecia previously flushed with a lethal amount of paraquat could not be rescued by later addition of these chemicals (data not shown).

From the opposite point of view, lecithin-rich or casein-rich biological materials were also found to be a positive additive for removing paraquat. As first aid for the inadvertent ingestion of a lipophilic toxicant, it is well known that the immediate drinking of milk is beneficial to retard absorption of the chemical(s) in the digestive ducts. Similarly, in a case of mistakenly drinking paraquat (hydrophilic), drinking milk (casein-rich) immediately might reduce the drug's lethal effects or reduce the amount of paraquat absorbed through the digestive ducts until stomach lavage treatment can be done in a hospital.⁸⁻⁹⁾ Experiments on this are in progress. Among some analogous dye chemicals involving the sulfonic group, trypan blue alone was effective (Table II). The reason is uncertain, however, it is often possible that a delicate difference in chemical structure will lead to a large difference in toxicity (methylparathion, high; fenitrothion, low toxicity) or in chemical reaction (ephedrine, negative detection; methamphetamine, positive detection with a reagent mixture of sodium nitroprusside, acetoaldehyde, and sodium carbonate).

Therefore, trypan blue offers a hint for the development of an antidote⁷⁾ against paraquat poisoning. Results of preliminary experiment showed that pure paraquat (methylviologen hydrate, Wako Pure Chemical Industries, Ltd. Osaka) killed paramecium under the same conditions as in the present experiments (use of 1000 times diluted Gramoxone[®]). Actually, it is usually not pure paraquat but Gramoxone[®] which is erroneously ingested. It has been generally stated that toxicity of a compound is multiplicatively or arithmetically strengthened by the coexistence of certain chemicals. Gramoxone[®] was employed to evaluate the toxicity of paraquat to paramecium in conformity with actual accidental ingestion. Gramoxone[®] contains the following ingredients as preventives¹⁰⁾: neutral surfactant (polyoxyethylene system, 3%), acidic dye material (0.15%), odorant (alkylpyridine system, 1%), emetic (0.05%), and a bitter material (denatonium benzoate, 0.005%). A paraquat-free effluent obtained after passing Gramoxone[®] through a Whatman CM-52 column contained the neutral surfactant and dye materials, but did not kill paramecium (data not shown). In a preliminary experiment, general acidic dye materials were harmless to paramecium (data not shown). It is uncertain how toxic to paramecium the other ingredients are. The evaluation of toxicity of paraquat to paramecium, however, was executed so as to include unknown effects of the other ingredients in conformity with the toxicity of Gramoxone[®].

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