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Binding of Paraquat with Water-Insoluble Tissue Preparations by Sephadex G-25 Thin-Layer Chromatography¹⁾

HIDEO ISONO and HARUYO SASAKI

*Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko-machi, Tsukui-gun,
Kanagawa 199-01, Japan*

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The present study was aimed at monitoring tissue materials for their binding ability toward paraquat (PQ: *N,N'*-dimethyl- γ,γ' -dipiridilium dichloride). Mixtures of PQ with various water-insoluble tissue samples were chromatographed on a Sephadex G-25 thin-layer plate. On the chromatographed plate covered with a sheet of cellophane was spread a solution of sodium hydrosulfite in 1 *N* sodium hydroxide solution for the detection of PQ as a blue spot. PQ remaining in the original location was considered to be in a state of binding with the tissue materials. Such tissue samples as brain, testis, thymus, pancreas, and submaxillary gland were found to have high ability to bind PQ. On the contrary, Harderian gland, cecum, and seminal vesicle sample, *etc.* failed to exert any binding ability. This binding mode was suggested to be based on ion exchange reaction, and largely governed by factors of ion strength and pH value of medium, and the fat content in tissue materials.

Keywords — paraquat; animal tissue material; ion exchange reaction; gel permeation thin-layer chromatography

Introduction

The herbicide paraquat (PQ: *N,N'*-dimethyl- γ,γ' -dipiridilium dichloride), is well known to cause serious lesions²⁻⁴⁾ in such mammalian tissues as the lung and kidney. The absorption of PQ through the digestive ducts and its excretion in the urine,⁵⁾ however, are not as well known. This implies that PQ can interact with such polar materials as protein^{6,7)} and other tissue materials.⁸⁾ The present study attempted to observe the binding of PQ with tissue materials by employing thin-layer chromatography on a Sephadex G-25 plate.^{9,10)} The following new technique was devised for detection of PQ on the gel plate: A chromatographed gel plate was tightly covered with a sheet of cellophane and stained blue for PQ with sodium hydrosulfite. This technique allowed the direct detection of PQ on the gel plate without disturbing the gel surface. Many kinds of tissue samples from rat were monitored

for their PQ-binding abilities.

Materials and Methods

Materials — Gramoxone® (24% paraquat aqueous solution) was purchased from Takeda Industrial Chemicals Co., Ltd., Osaka, Japan. Other reagents used were of analytical grade from Wako Pure Chemical Industries, Co., Ltd. Sephadex® G-25 was purchased from Pharmacia Fine Chemicals AB Uppsala Sweden. Adhesives: Shinetsu Silicon® was from Shinetsu Chemical Industries, Ltd., Japan; and Arabic Yamato® from Yamato Co., Ltd., Japan. Filter papers: Type Nos. 131 and 526 were from Toyo Roshi Co., Ltd., Tokyo, Japan. Cellophane: A sheet type, available for general wrapping. Animals: Several male Wistar strain rats (*ca.* 250 g body weight) and a female Japanese white strain rabbit (*ca.* 3.5 kg) were from Sankyo Laboratory Co., Tokyo, Japan. The criterion color table used was that officially authorized.

Preparation of Water-Insoluble Tissue Samples — As shown in Chart 1, five male Wistar strain rats were exsanguinated by decapitation. Various tissues or organs were washed with water and blotted with paper before weighing. First, one quarter of each tissue or organ was conventionally homogenized in two volumes of water using a Teflon-glass homogenizer. The whole homogenate was harvested and one half was utilized as homogenate sample A (see Table I). The other half was subjected to three cycles of a freezing (-20°C)–thawing (4°C) process. The precipitates appearing were collected by centrifugation at $900 \times g$ for 5 min. This was called water-precipitate sample D. Second, two quarters of each tissue or organ were similarly homogenized with 50 volumes of water and passed through a No. 131 filter paper. The residue on the paper was thoroughly washed with water. This was called solid material sample B (membrane fragment, plexus, fiber, *etc.*). The filtrate was adjusted to pH 5.0 with acetic acid. The resultant precipitate was collected by centrifugation as above and thoroughly washed with water; this was called acid precipitate sample C. Stomach, small intestine, cecum, and colon tissues were each divided into mucosal and muscular parts. Both parts were separately subjected to the preparation of sample B. Filtrates from both these mixtures were then combined and subjected to the preparation of sample C. Third, the final one quarter of each tissue or organ was similarly homogenized in two volumes of 0.1 M potassium phosphate buffer solution (pH 7.4). The entire homogenate was subjected to three cycles of a freezing–thawing process as above. After centrifugation ($900 \times g$, 5 min), the precipitate-rich layer was collected as buffer precipitate sample E.

Gel Permeation Thin-Layer Chromatography (TLC) — Sephadex G-25 (8 g) was allowed to swell in 40 ml of water overnight at room temperature. All of the slurry was poured onto a glass plate (net square: 19×19 cm, with banks) and evenly spread. The banks were continuously constructed by sticking several pieces of glass (1

mm thick, 5 mm wide) to the edges of a glass plate (20×20 cm, 1 mm thick) with silicon adhesive. Less than $20 \mu\text{l}$ of aliquots of tissue sample suspension spiked with PQ were applied to the gel plate along a starting line 3 cm from one edge. The plate was horizontally set and the edge of the sample side was linked to a developing solvent in a reservoir *via* a bridge of paper (5 cm long, 19 cm wide, and No. 526). The opposite edge touched another paper (5 cm long, 19 cm wide, No. 526, and triangle shape), which hung down to allow the solvent to drop out. The level of the solvent surface of the reservoir was made equally to the gel surface. The plate was developed for 2 h at room temperature. The bank was coated with the adhesive (Arabic Yamato®) to accomplish the following covering process. The gel surface was completely covered with a sheet of cellophane (23×23 cm) previously soaked in water so as to avoid trapping air between the cellophane and the gel surface. At this stage, the plate was photographed to record the background picture. Next, 0.5 g of sodium hydrosulfite was dissolved in 15–20 ml of 1 N sodium hydroxide solution just before use, then poured immediately onto the covered gel plate and then gently spread over the entire surface. When blue spots of PQ appeared in a minute or so, the plate was immediately photographed again to record the herbicide's mobility.

Chromatography of PQ on Column of Defatted Tissue Material — Various whole homogenate samples of rat tissues (1:2, v/v in water) were each mixed with chloroform and methanol at the ratio of 4:5:10, v/v/v, respectively. It was allowed to stand overnight at room temperature and passed through a No. 131 filter paper. The residue on the paper was thoroughly washed in the order of water–chloroform–methanol (4:5:10, v/v/v), methanol, and *ca.* 3×10^{-3} M ammonia solution (pH 8–9). The defatted material was suspended in the same ammonia solution and packed in a glass tube plugged with glass wool in a column with a diameter of 0.9 cm ϕ and 0.5 cm high. Gramoxone® was diluted 10 times with water and $10 \mu\text{l}$ of it was mixed with 0.2 ml

of the same ammonia solution. This was applied to the minicolumn. The column was washed with the same ammonia solution and eluted with *ca.* 7.5×10^{-3} M acetic acid (pH 4–5) to give 0.2-ml fractions, beginning with the sample application. A 20- μ l aliquot of each fraction was spotted on a No. 526 filter paper and sprayed with 1 N sodium hydroxide solution followed by heating with a hair dryer to detect PQ as a blue spot.¹¹⁾ The intensity of the blue was compared with the criterion color table.

Staining of Tissue Section with PQ — A tissue sample was frozen at -20°C and cut into sagittal or transverse sections (less than 0.1 mm thick). The section was dipped in 100-fold diluted Gramoxone® with water for 2–3 h at room temperature. The medium was changed to a larger volume of water and allowed to stand overnight in an ice-bath. After removal of the medium, a 2.5% sodium hydrosulfite solution in a 1 N sodium hydroxide solution was gently dropped onto the section. The blue-colored section was

immediately photographed. After disappearance of the blue, it was photographed again to record the background.

Results and Discussion

Interaction of PQ with Water-Insoluble Tissue Sample Material

First, 20 μ l (*ca.* 5 mg dry weight) of homogenate sample A was mixed with 2 μ l of 100-fold diluted Gramoxone® and chromatographed on a Sephadex G-25 thin-layer plate. After the treatment with sodium hydrosulfite, the chromatograms gave both or one or the other of two blue spots: a tailing one at a site 5.5 cm away and an immovable one at the position of origin (Fig. 1). The former was called a 5.5-cm migration blue and the latter a zero-migration blue. The standard Gramoxone® alone migrated 2.7 cm for a full migration length of 16 cm. The 5.5-cm migration blue implied that PQ was attracted by some much more movable water soluble materials in sample A. The zero-migration blue demonstrat-

Chart 1. Procedures for the Preparation of Rat Tissue Samples

ed that PQ was bound by water-insoluble materials such as membrane fragments, plexus, fibers, or water-insoluble sediment derived from some water-soluble materials. Thus, a visual evaluation of the zero-migration blue was found most convenient to determine whether PQ bound to tissue materials or not. Intensity of the zero-migration blue is summarized for the different tissue samples in column A-I of Table I. This evaluation method was employed in the remainder of experiments. In the combination system of homogenate sample A and water (I) used for gel plate preparation and development, Harderian gland¹²⁻¹³, adrenal, cecum and mesenterium samples did not exert a PQ-binding ability. The others did show the ability. Brain, colonic mucosa and testis samples were particularly potent.

Second, as a definite tissue sample, sample B was similarly tested for PQ-binding ability changing the system in pH value or salt concentration. Evaluation of the zero-migration blue is summarized in columns B-I, II, III, and IV of Table I. The difference between the two profiles by systems B-I and B-IV suggested the PQ-binding ability of tissue materials to be lessened depending on the kinds of salts. The difference between system B-III and B-IV suggested that the binding ability was also lessened by acidic condition. In combination with sample B and water (I), Harderian gland (plexus and membrane), cecum (muscular fiber), mesenterium (plexus) and seminal vesicle (contents) showed negative binding, while the others did show the ability to a greater or lesser degree. Thymus (plexus), muscular fiber, and colonic mucosa were particularly significant. When the water was changed to an ammonia alkaline solution (pH 8-9), Harderian gland (plexus and membrane) exerted a little of the ability. On the contrary, stomach (muscular fiber), colonic muscular fiber, seminal vesicle (bag) lost the ability. When the water was changed to 0.1 M potassium phosphate buffer solution (pH 6.5) (III), most of the samples lost the ability; only the brain sample retained it slightly. When the above buffer solution (III) was changed in pH value from 6.5 to 7.4 (solvent IV), brain (precipi-

tate), thymus (plexus), pancreas (plexus), and muscular fiber recovered the ability significantly.

Third, for determination of PQ-binding ability of water-soluble material as the zero-migration blue, it was precipitated for addition to water-insoluble material by a method of freezing-thawing in a medium of water for sample D or 0.1 M potassium phosphate buffer solution (pH 7.4) for sample E. Both samples D and E were similarly tested in a salty environment of 0.1 M potassium phosphate buffer solution (pH 7.4), resembling a physiological fluid. As shown in columns D-IV and E-IV of Table I, the additional precipitates

Fig. 1. Sephadex G-25 Thin-Layer Chromatograms of Tissue Whole Homogenate Sample A (in Table I) Spiked with Paraquat (PQ)

Upper: before color-development with sodium hydrosulfite.
Lower: immediately after the development.

Samples loaded: mixture of rat tissue whole homogenate (20 μ l, 1:2 v/v in water) and 2 μ l of 100-fold diluted Gramoxone® (24% paraquat dichloride).

TLC: 8 g of Sephadex G-25 in 40 ml of water per plate (net square of 19 $\times$ 19 cm); development for ca. 2 h with a 10-ml flow of water at room temperature.

Star-signed samples significantly gave the zero-migration blue. The photograph shows part of the samples mentioned in the text.

TABLE I. Paraquat-Binding Ability of Water-Insoluble Tissue Sample Materials

Sample mode		A		B		C		D	E
Developing solvent		I	I	II	III	IV	IV	IV	IV
Tissue species	Brain	3+	2+	2+	+	2+	+	2+	2+
	Eyeball	+	2+	+	—	—	2+	—	—
	Harderian gland	—	—	+	—	—	—	—	—
	Submaxillary gland	+	2+	2+	—	+	2+	+	+
	Lung	+	+	3+	—	—	+	+	+
	Thymus	+	4+	2+	—	2+	2+	+	2+
	Heart	+	2+	+	—	+	+	+	+
	Blood	/	+	+	—	—	—	/	/
	Liver	+	+	2+	—	—	+	+	+
	Kidney	+	2+	2+	—	+	+	2+	2+
	Adrenal	—	+	+	—	—	—	—	—
	Stomach ^{a)}	+	2+	+	—	+	+	+	2+
			+	—	—	—	—	—	—
	Small intestine ^{a)}	+	+	2+	—	—	+	+	+
			+	—	—	—	—	—	—
	Cecum ^{a)}	—	+	+	—	—	—	—	—
			—	—	—	—	—	—	—
	Colon ^{a)}	2+	3+	2+	—	+	2+	+	+
			+	—	—	—	—	—	—
	Spleen	+	+	3+	—	—	+	—	+
	Pancreas	+	2+	2+	—	2+	2+	2+	3+
	Mesenterium	—	—	—	—	—	—	+	+
	Testis	2+	2+	3+	—	+	2+	+	+
	Seminal vesicle	+	—	—	—	—	—	—	—
	Prostate	+	+	2+	—	+	+	2+	+
	Muscle	+	4+	2+	—	2+	2+	+	+

Sample species of tissues: A, whole homogenate; B, solid material; C, acid-precipitate; D, water-precipitate; and E, buffer-precipitate. Solvent for gel preparation and development: I, pure water; II, pH 8–9 ammonia alkaline solution; III, pH 6.5, 0.1 MKH₂PO₄-Na₂HPO₄ buffer solution; and IV, pH 7.4 the same buffer solution as III. Assessment of the blue at the origin of TLC: —, negative; +, weak; 2+, ordinary; 3+, strong; and 4+ very strong. a): upper, mucosal part; and lower, muscular part.

from kidney and pancreas samples were suggested to enhance the zero-migration blue, even in the presence of salt, more than the corresponding results in column A-I. This implied that the water-soluble tissue materials were also responsible for the PQ-binding. Consequently, sample C was prepared from water-soluble material alone and similarly tested for the binding in the presence of salt. Almost all of the samples showed the zero-migration blue as shown in column C-IV, demonstrating that the water-soluble materials also had the ability to bind PQ. This kind of water-soluble material would, therefore, in part attract PQ to give the 5.5-cm migration blue (standard PQ: 2.7 cm), which was previously observed by Sephadex G-25 TLC of the homogenate sample

A spiked with PQ.

Influence of Fat on Binding of PQ with Water-Insoluble Tissue Materials

Figure 1 shows chromatographic profiles of PQ on a minicolumn of defatted tissue materials. Most of the defatted tissue samples were found to have the ability to bind PQ in a medium of ammonia alkaline solution (pH 8–9) and to release it in a medium of acetic acid solution (pH 4–5). This alkaline solution was more effective than water. When the eluant was altered from the above alkaline solution to a salt-rich one like 0.1 M potassium phosphate buffer solution (pH 7.4–8.0), PQ loaded on the column was completely eluted in the early fractions of eluate (data not shown).

The Harderian gland sample failed to demonstrate PQ-binding ability under any experimental system (except system B-II) of Table I. However, this tissue sample was found to have the ability after the removal of fat. Even adipose tissue showed a little of it after the removal of fat. This chromatographic PQ-binding behavior of the defatted materials coincided with the behavior of the solid material sample B (Table I) for thymus, kidney, pancreas, *etc.*, which was observed between systems B-III (acidic) and B-IV (alka-

line) in Table I. This agreement implied that the defatting treatment probably did not cause the loss of the intrinsic PQ-binding ability of tissue, but provided the ability. These elution profiles suggested that the tissue materials acted as a cation exchanger against PQ. The defatting effect implied that PQ-binding of tissue is dominated by the presence of fat.

Influence of Salts on Migration of PQ by Sephadex G-25 TLC

Several kinds of salt (20 μ l of 0.1 M solution as listed in Table II) were each mixed with 2 μ l of 100-fold diluted Gramoxone®. They were chromatographed on a Sephadex G-25 plate using water for the gel plate conditioning and as developer. As shown in Table II, the presence of H_3BO_3 or $Na_2B_4O_7$ did not affect the mobility of PQ and permitted it to fall within a migration distance of 1.5–2.0 cm (maximum migration distance: 16 cm). This behavior was the same without salt. Curiously, the other salts tested carried PQ to a distance of 10–13 cm. This high mobility may be

Fig. 2. Elution Profiles of Paraquat from a Column of Defatted Water-Insoluble Tissue Sample Materials

Defatted Materials as adsorbent: precipitates obtainable by mixing tissue whole homogenate (1:2, w/v in water) with chloroform and methanol at the ratio of 4:5:10, v/v/v, respectively. Column: material 0.9 cm in diameter and 0.5 cm high, which was equilibrated with ammonia alkaline solution (pH 8–9). Paraquat loaded: 10 μ l of Gramoxone® (24% paraquat dichloride) diluted 10-fold with water. Detection of paraquat as a blue spot: 20 μ l of each eluate (200 μ l) was spotted on a filter paper and sprayed with 1 N NaOH solution and heated by a hair dryer. The density of the blue was compared with a criterion color table.

TABLE II. Influence of Salts on Migration of Paraquat on a Sephadex G-25 Thin-Layer Chromatoplate

Salt	Migration(cm)
Water	2.3
H_3BO_3	2.0
$Na_2B_4O_7$	1.5
NaCl	11.5
KCl	11.5
NH_4Cl	12.5
$(NH_4)_2SO_4$	14.1
$NaHCO_3$	12.5
Na_2CO_3	11.5
KH_2PO_4 - Na_2HPO_4 buffer pH7.4	14.1
KH_2PO_4 - Na_2HPO_4 buffer pH6.8	14.1
Malonic acid	10.3
Sodium citrate	13.0

Sample: mixture of 20 μ l each of 0.1 M salt solution listed and 2 μ l of Gramoxone® (24% paraquat dichloride) diluted 100-fold with water. TLC: 8 g of Sephadex G-25 granule/40 ml water for a plate 19 $\times$ 19 cm square; 16 cm for maximum movable distance; development for 2 h at room temperature with pure water; and color development with 2.5% sodium hydrosulfite in 1 N NaOH solution.

interpreted as follows: The anions, except BO_3^{3-} or $\text{B}_4\text{O}_7^{2-}$, were repelled by the negative charge of Sephadex G-25 material, which is known to be a side effect. The repelled anion attracted PQ and carried it away. Therefore, anions also seemed to be responsible for the appearance of the 5.5-cm migration blue as discussed for the acid precipitate sample C.

***In Vitro* Binding of PQ to Colonal Tissue Section**

Photograph A in Fig. 3 shows a transverse section of a rabbit ascending colon. This reveals an external muscular layer (white image), inner muscular layer (black), submucosa (white), muscularis mucosa (invisible), lamina propria (basal zone of pleat-like image), and epidermal cell layer (pleat-like image). This section was stained with PQ and color-developed. The resultant photograph B showed an additional circular black

image (actually blue image), which suggested that PQ was very familiar with mucosa (lamina propria and epidermal cells). This result agrees with the PQ-binding results of the colonal samples summarized in columns B-I and B-II of Table I, and this agreement would support that the PQ-binding ability of tissue can be assessed by the evaluation of the zero-migration blue.

A binding or an interaction of a trace amount of PQ with physiologically potential tissue materials is more noticeable than a high PQ-tissue accumulation alone, because high PQ-tissue accumulation is not always able to account for tissue lesions.¹⁴⁾ It is generally difficult to directly observe the binding or the interaction without any treatment. The present study employed Sephadex G-25 TLC for this purpose and concluded that 1) some water-insoluble tissue materials such as membrane fragments, plexus, fibers, and a form of sediment generated from water soluble materials has the ability to bind PQ; 2) some water-soluble materials such as anionic chemicals has the ability to interact with PQ, attracting it and

Fig. 3. In Vitro Binding Profile of Paraquat with Rabbit Colonal Tissue Section

Photograph: A, intact section; B, the section stained with paraquat and colored blue with 2.5% sodium hydrosulfite in 1 N NaOH solution; and C, the section proposed after the disappearance of the blue. EE: exterior muscular layer. IM: inner muscular layer. SM: submucosa. MM: muscularis mucosae. LP: lamina propria. EC: epidermal cell layer.

releasing the binding; and 3) the binding mode was based on an ion exchange reaction and dependent on the pH value in the medium. This binding profile resembled that of some acidic dyes which can be bound by a colorless woolen material in an acetic acid solution and released from the binding in an ammonia alkaline solution. The characteristic high PQ-binding ability of brain, pancreas, and prostate tissue preparations was associated with the following human anatomical results: congestion in the brain^{2,4)}; degradation of the pancreas⁴⁾; and noticeable PQ-distribution in the

prostate.⁵⁾ These findings suggested that a molecular investigation of PQ-binding or PQ-interaction with tissue materials would contribute to some degree to elucidation of the primary mechanism(s) of urinary excretion delay of PQ,⁵⁾ PQ-tissue accumulation in, for instance, lung and kidney,¹⁴⁻¹⁶⁾ lung tissue-lesion by PQ,¹⁷⁾ enzyme interference by PQ (*e.g.*, cholinesterase,⁷⁾ cell membrane transportation of PQ with or without carrier, *etc.* Finally, elucidation of these mechanisms could lead to the detoxication of PQ.^{18,19)}

References

- 1) Presented at the 109th annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1989.
- 2) S. Tsunenari and H. Mutoh, *Nippon Hoigaku Zasshi*, **29**, 88 (1975).
- 3) M.H. Litchfield, J.W. Daniel and S. Longshaw, *Toxicology*, **1**, 155 (1973).
- 4) T. Fujikura and H. Takizawa, *Nippon Hoigaku Kaishi*, **42**, 248 (1988).
- 5) M. Misago, Y. Senda, K. Yu, Y. Hanaoka, O. Hasegawa, Y. Nakajima, A. Kuroiwa, A. Kajiki, S. Harada, Y. Shiroto and A. Horie, *Nippon Kyobu Rinsho*, **43**, 298 (1984).
- 6) R. Tanaka, S. Fujisawa and K. Nakai, *J. Toxic. Sci.*, **6**, 1 (1981).
- 7) T. Shinohara and Y. Seto, *Agric. Biol. Chem.*, **50**, 255 (1986).
- 8) T.M. Sullivan and M.R. Montgomery, *Drug Metabolism and Disposition*, **11**, 526 (1983).
- 9) B.G. Johansson and L. Rymo, *Acta. Chem. Scand.*, **16**, 2067 (1962).
- 10) B.G. Johansson and L. Rymo, *Acta. Chem. Scand.*, **18**, 217 (1964).
- 11) H. Isono, Y. Sakaguchi, H. Katoh and S. Miyaura, *Eisei Kagaku*, **32**, 300 (1986).
- 12) J.J. Harder, *Acta Eruditorium Lipsiae*, **1694** 49.
- 13) T. Sakai, *Arch. Histol. Jpn.*, **44**, 299 (1981).
- 14) M. Shinozaki, N. Tokunaga and Y. Kobayashi, *Nippon Kyobu*, **46**, 258 (1987).
- 15) C.W. Sharp, O. Ottolenghi and H.S. Posner, *Toxicol. Appl. Pharmacol.*, **22**, 241 (1972).
- 16) E. Kurisaki and H. Sato, *Forensic Sci. Int.*, **14**, 165 (1979).
- 17) H. Witsch, S. Cacew, K. Hirai and M.M. Cote, *Chem. Biol. Interact.*, **19**, 143 (1977).
- 18) S. Ukai, K. Nagai, T. Kiho, T. Tsuchiya and Y. Nochida, *J. Pharmacobio-Dyn.*, **10**, 682 (1987).
- 19) E. Kurisaki, *Eisei Kagaku*, **35**, 261 (1989).